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Pichinde Virus (PIC) is the animal model for human Lassa Fever. The pathogenic mechanisms were examined by comparing a virulent guinea pig (GP) adapted PIC strain, adPIC, and the GP avirulent prototype PIC strain PIC3739. adPIC replicated to higher titers than PIC3739 in macrophages infected in vivo and in vitro. Lethal infections were associated with pronounced shifts in visceral macrophage distribution. Tumor necrosis factor (TNF) - like bioactivity levels increased in the serum of adPIC but not PIC3739 infected GPs. Spleen TNF activity rose during the first week after infection by both viral strains. However, neither in vitro nor in vivo infected macrophages by either virus produced more TNF. Peritoneal macrophages explanted from adPIC infected GP (day 11) showed augmented TNF production after LPS stimulation as compared with PIC3739 infected or uninfected cells. We now theorize that TNF secretion patterns relate to the level of virus infection or replication and there appear to be structural differences in these two viruses that may account for different pathogenicities. Characterization of these structural differences will be important in developing potential model vaccine strains. Finally, results are also presented that support the theory that adPIC infection is associated with a progressive suppression of lymphocyte responses.

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ABSTRACT

Pichinde Virus (PIC) is a new world arenavirus which is the basis of a guinea pig (GP) model for human Lassa Fever. We have investigated macrophage and T-lymphocyte functions to gain insight into immunity and pathogenesis in this model. Pathogenesis studies are based on the comparison of a virulent GP passage-adapted PIC strain, adPIC, and the prototype PIC strain, PIC3739, which is avirulent in GP. adPIC replicated to higher titers than PIC3739 in target macrophages in both in vivo and in vitro infections. Lethal infection with adPIC was associated with pronounced shifts in visceral macrophage distribution. Tumor necrosis factor-like bioactivity was found in the serum of adPIC-infected, but not PIC3739-infected guinea pigs, where TNF levels rose during the course of infection. TNF-like activity appeared in the spleen in animals infected with either PIC strain, peaking during the first week of infection. TNF mRNA was increased in adPIC infected spleens at day six. There was no increase in constitutive TNF production in macrophages infected in vivo or in vitro with either PIC strain. However, peritoneal macrophages (PM) explanted from adPIC infected GP at day 11 showed augmented TNF production after LPS stimulation compared to control uninfected macrophages or macrophages from PIC3739 infected animals. In contrast, spleen macrophages from adPIC infected animals showed suppression of TNF production after LPS stimulation. After in vitro infection with PIC, PM showed a complex pattern of change in LPS-inducible TNF secretion; we theorize this pattern of TNF secretion relates to level of virus infection of a population of cells, hence differences in TNF regulation in the two viral systems may correlate with rates of viral replication. Preliminary evidence suggests that there are structural differences between the two strains of Pichinde virus. The nature of these differences remain to be determined, as is their role of the different pathogenicities.

Immunosuppression has been associated with other arenavirus infections. To explore the possibility that immune dysfunction occurs in strain 13 guinea pig infected with adPIC, we have examined the mitogen responses of mononuclear cells isolated from the spleens and peripheral blood of adPIC infected strain 13 guinea pigs. Mononuclear cells isolated from adPIC infected guinea pigs manifested markedly depressed proliferative responses to concanavalin A (con A) and phytohemagglutinin (PHA) compared to uninfected controls. No change in the kinetics of response to mitogen was apparent in cells isolated from infected animals. Mixing experiments showed that plastic adherence-isolated macrophages from adPIC infected animals were capable of supporting normal proliferative responses of nylon wool-isolated T-lymphocytes from control animals. Conversely, nylon wool-isolated T-lymphocytes from infected animals showed dramatically diminished responses to mitogen in the presence of macrophages from control animals. These results are consistent with the conclusion that adPIC infection of susceptible guinea pigs is associated with a progressive suppression of lymphocyte responses.

INTRODUCTION

Human viral hemorrhagic fevers are acute, fulminating diseases, generally characterized by fever, hemorrhage, and shock. The etiologic agents include a range of geographically restricted viruses (Centers for Disease Control, 1988). Research in pathogenesis of viral hemorrhagic fevers has been hampered by the hazardous nature of these agents. The Lassa Virus is a member of the family arenaviridae which also includes Junin and Machupo viruses, the etiologic agents of Argentine and Bolivian Hemorrhagic Fever. Lassa Fever is endemic in West Africa, where it causes approximately 200,000 - 400,000 infections and 5,000 deaths annually (McCormick, 1987). An animal model for Lassa Fever utilizes a related virus, Pichinde virus (PIC), a New World arenavirus of the Tacaribe group (Trapido, 1971). A PIC strain passage-adapted for virulence in guinea pigs (adPIC) is not pathogenic for humans. Inbred strain 13 guinea pigs are highly susceptible to infection with this virulent derivative of PIC (adPIC) and develop a severe illness characterized by fever, wasting, and viral replication in many organs before death in the third week post-inoculation (Jahrling, 1981). The relevance of this model to human Lassa Fever stems principally from the relatedness of the viruses, and the relative paucity of histopathologic lesions in virally infected guinea pigs despite the fulminant disease course. Fatal Lassa fever accounts for 1-2% of cases of Lassa fever (Cummins, 1990), and manifests fever, facial edema, pleural effusion, adult respiratory distress syndrome, mild hemorrhagic tendencies, and "shock". Some have suggested clinical similarities to endotoxic shock (McCormick, 1986). However, pathologic findings in such fulminant cases are minimal, non-specific and do not suggest a pathogenetic sequence. Significant lesions are largely restricted to the liver in human Lassa Fever and consist of spotty hepatocellular necrosis with minimal accompanying inflammation (Winn, 1975). Similar changes have been described in livers from adPIC infected guinea pigs (Lucia, 1990). Many authors have stressed that the degree of liver damage is not sufficient to account for death. This discrepancy between the severe clinical course and the scanty histopathologic evidence of cellular damage has led to speculations that Lassa virus pathogenesis relates to either disturbance in cellular function or induction of toxic or pharmacologic mediators of fever, shock, and vascular collapse. These observations together with indications that arenaviruses are capable of infecting cells of the reticuloendothelial system (Murphy, 1977) encouraged us to investigate the role of the monokine tumor necrosis factor α (cachectin) (TNFα) in the pathogenesis of adPIC disease in guinea pigs. TNFα has a variety of effects including mediation of endotoxic shock, cachexia of chronic disease, antiviral properties, and others. In these studies, we have sought to delineate the degree of macrophage involvement in experimental adPIC infection and to document the presence of TNF activity in serum, organ homogenates and culture supernatants from macrophages explanted from adPIC infected animals. We describe the use of the avirulent, prototype PIC strain, PIC3739, as biologic reference for physiologic, protective monokine responses to viral infection.

Immunosuppression has been previously associated with adPIC infection and other arenaviruses. Decreased or ineffective humoral immune responses have been observed in Junin (Frigerio, 1977 and Carballal, 1981), Lassa and PIC infections (Peters, 1987). Decreased mitogenic responses have been observed in Junin infections (Frigerio, 1977), and decreased ability to develop effective cytotoxic T-lymphocytes have been observed in Lassa Virus infections (Peters, 1987). Based on these observations we have explored the extent to which adPIC causes immunosuppression in strain 13 guinea pigs, and the potential mechanisms by which adPIC induces this dysfunction. A well known indicator

of in vivo immune function is the in vitro responsiveness of lymphocytes to mitogenic stimulation (Liener, 1986). For this reason, we began the process of characterizing T-lymphocyte function in guinea pigs infected with adPIC by examining in vitro splenocyte and peripheral blood mononuclear cell responses to mitogen. Subsequent to this work, we sought to characterize the defects associated with the diminished responsiveness of lymphocytes to mitogen.

MATERIALS AND METHODS

Virus:

Two Pichinde (PIC) strains were utilized. A virulent derivative of PIC AN4763 was developed by Jahrling et al. (1981), after serial guinea pig passages. This strain is hereafter termed adPIC. The adPIC stock, originally derived from Jahrling's passage adapted strain, was obtained from Dr. Dorian Coppenhaver, Dept. of Microbiology, UTMB, as spleen homogenate from the fifteenth guinea pig passage. A less virulent PIC strain, CoAN3739, was obtained from ATCC as 10% suckling mouse brain suspension, and is hereafter abbreviated PIC3739. Viral stocks were generated in strain 13 guinea pigs as follows: strain 13 guinea pigs were inoculated intraperitoneally with approximately 10^{3-4} PFU of the fifteenth animal passage of adPIC or suckling mouse brain suspension of PIC3739. Six days after inoculation, spleens from infected animals were aseptically removed, homogenized in a 10% (w:v) mixture with supplemented RPMI 1640, and then stored in aliquots at -70°C. The adPIC stocks from the 16th and 17th spleen passages and PIC3739 stocks from the first guinea pig passage were used in these experiments; stock viral titers ranged between 3 and $6x10^5$ plaque forming units (pfu) per ml.

Concentrated viral suspensions were generated as described previously (Young, 1983). Briefly, lightly confluent monolayers of Vero cells were inoculated with PIC stock diluted to achieve an approximate multiplicity of infection of 0.04. Virus was adsorbed for 60 mins at 37°C, then flasks were refed with Earle's modified minimal essential medium (EME) (Gibco) supplemented with 2% fetal bovine serum (Hyclone Laboratories, less than 0.07 ng endotoxin per ml) (FBS), 2mM L-glutamine, 10mM Hepes buffer, and gentamicin. After 24 hrs, monolayers were washed twice with Hanks Balanced Salt solution (HBSS) and refed with EME/2% FBS. Forty-eight hrs post-inoculation, tissue culture supernatants were harvested and clarified by centrifugation at 1800xg for 30 mins at 4°C. Clarified culture fluids were adjusted to 0.4M NaCl and 6% polyethylene glycol (average MW 8000, Sigma). Precipitation was carried out at 4°C for 2 hrs. The suspension was centrifuged at 10,000xg for 20 mins (4°C), the pellet was resuspended in GNTE buffer (0.2M glycine, 0.2M NaCl, 0.02M Tris-HCl pH 7.8, 0.002 M EDTA). The material was sonicated for 37 seconds at 32 kHz in an ice bath, then centrifuged at 3000xg for 10 mins to remove residual particulate material. Supernatants were collected and stored at -70°C. This procedure resulted in 100-fold concentration of virus over the initial tissue culture media. Culture fluids from mock infected Vero cells were treated identically and used as control in in vitro macrophage infection and lymphocyte proliferation experiments.

Viral Plaque Assay: Virus was quantitated in a standard plaque assay on Vero cells as described previously, (Lucia, 1990), except that Vero cells were exposed to 2500 rads before plating. Briefly, irradiated Vero cells were incubated in supplemented EME overnight in 24 well tissue culture plates at 37°C in 5% CO_2. Diluted 0.1 ml samples of virus were prepared in quadruplicate and were added to the wells. After incubation

for 1 hr, at 37°C and 5% CO_2 viral inocula were removed and, 1 ml of methyl cellulose overlay media containing 30 μg/ml gentamicin, and 2% FBS was added to the wells. The plates were then incubated at 37°C in 5% CO_2 for 6 days and then 1 ml of a 0.01% neutral red (Gibco) solution in overlay media was added. Plaques were counted 8-12 hrs later.

Guinea Pigs: Strain 2 guinea pigs were obtained from University of Texas, M.D. Anderson Cancer Center, Bastrop, TX, and outbred Hartley guinea pigs were obtained from Harlan Sprague Dawley. Strain 13 guinea pigs (350-600 grams) obtained from Crest Caviary (Mariposa, CA) were free of specific detectable pathogens, and were housed in microisolator cages. For macrophage studies, guinea pigs were inoculated intraperitoneally with $2\text{-}4\text{x}10^3$ PFU virus in spleen homogenate or with diluent alone on day 0. For studies of immunosuppression, all test animals were infected by intraperitoneal injection of $2\text{x}10^4$ PFU of passage number 16 adPIC in 1.0 ml of RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics. Daily weights and rectal temperatures were recorded. Guinea pigs were termed moribund and sacrificed when they had lost 25% of their initial body weight and rectal temperature fell below 39.8°C. Guinea pigs were euthanatized by exsanguination by cardiac puncture under ketamine/xylazine anesthesia. Comprehensive serology profiles were performed on selected guinea pigs by Research Animal Diagnostic and Investigative Laboratory, University of Missouri, College of Veterinary Medicine, Columbia, MO. Bacterial cultures were performed by the Clinical Microbiology Service at University of Texas Medical Branch.

Macrophage Culture: Resident peritoneal macrophages were harvested by aseptic lavage of the peritoneal cavity with ice cold, calcium magnesium-free HBSS supplemented with gentamicin. Recovered cells were washed once with HBSS and resuspended in RPMI 1640 (Biofluids) supplemented with 10% fetal bovine serum (Hyclone, less than 0.07 ng endotoxin per ml), 2mM L-glutamine, and 30 μg/ml gentamicin sulfate. Cells were counted in a Coulter counter, and differential leukocyte counts were performed on Diff-Quick (Baxter S/P) stained cytospin preparations. These suspensions usually ranged between 65% and 90% macrophages based on morphology. Cells were cultured in 24 well plates (Gibco) at a concentration of 10^6 per ml. After an 8-16 hr incubation at 37°C in a humidified 5% CO_2 atmosphere, non-adherent cells were removed by washing plates 3 times with warmed media and cultures were refed with supplemented RPMI alone or media containing 10 μg/ml *E. coli* O111:B4 LPS (Difco). Supernatants were harvested 24 hrs later for assay of mediators. Alveolar macrophages were obtained by cannulation of the distal trachea and lavage of the lungs with ice-cold HBSS. Cells were washed once, counted and cultured as above. These populations normally consisted of >90% macrophages. For preparation of spleen macrophage cultures, single cell suspensions of spleen were made in HBSS, and washed once in HBSS. Total and differential cell counts were performed and cells were plated at 10^6 macrophages per ml. Non-adherent cells were removed by rinsing monolayers with media after 12-18 hr incubation at 37°C in 5% CO_2. Liver sinusoidal lining cells were obtained by modifications of previously described procedure (Rubinstein, 1987). Guinea pigs were anesthetized and treated with 2000 U heparin by intracardiac injection. The thoracic inferior vena cava and portal veins were cannulated, and the liver was perfused retrograde or antegrade at room temperature with calcium magnesium-free HBSS with gentamicin, followed by 0.5mM ethyleneglycol-bis-(ß-aminoethyl ether) N,N,N'N'-tetraacetic acid (EGTA) in phosphate buffered saline (PBS), PBS alone, and finally with 0.5 mg/ml collagenase A (Boehringer-Manheim) in HBSS. The liver was excised aseptically and minced in warmed (37°C) HBSS. The tissue was digested in HBSS containing 0.3% collagenase, 1% FBS, 0.004% DNase I

(Sigma), and 2.5mM Hepes buffer with gentle mixing at 37°C for 30 mins. The digested mixture was washed through nylon cloth with cold HBSS, and the suspension was centrifuged at 400xg for 10 mins at 4°C to pellet cells. Erythrocytes were lysed with 0.45% NaCl and cells were washed once more in HBSS. The suspension was overlaid on a discontinuous Percoll gradient (1.04 and 1.07 g/ml) and centrifuged at 400xg for 20 mins at 20°C. Cells at the interface between Percoll layers were harvested, washed twice in cold PBS, resuspended in complete culture media containing 10% FBS and antibiotics and plated at a concentration of 10^6 mononuclear cells per ml. Non-adherent cells were removed as described previously. By this technique, approximately 75% of resultant adherent cells were macrophages by immunofluorescence (see below). In some experiments, macrophages were cultured on sterile glass cover slips, treated as above, and fixed in acetone or 1% paraformaldehyde for immunofluorescence microscopy.

Infectious Center Assay: Macrophages from infected or uninfected control animals were plated in 24 well plates at varying concentrations. After washing to remove non-adherent cells and free virus, macrophages were overlaid with $2x10^5$ Vero cells previously treated with 2500 rads. The number of adherent macrophages was estimated visually for each separate culture. Wells were overlaid with media containing 0.5% agarose, and refed on day 6 with an agarose overlay containing 0.01% neutral red. Plaques were counted 12 hrs later. Results are reported as plaques (infectious centers) per 1000 adherent cells, based on the visual estimate of adherent cells. "Plaque lifts" were achieved by transferring putative viral antigens in plaque containing monolayers to nitrocellulose discs. Immunostaining for absorbed viral protein was performed using a guinea pig anti-adPIC antiserum; signal was amplified and developed using a Vectastain ABC peroxidase kit (Vector Lab).

In Vitro Infection of Macrophages: Mineral oil elicited peritoneal macrophages (PM) were obtained from healthy strain 13 guinea pig injected intraperitoneally 4 days prior to sacrifice with 10 cc mineral oil (Fisher). Macrophages were harvested as above and washed 3 times in cold HBSS to remove excess oil. Cells were divided into equal aliquots of about 40 million macrophages each, and pelleted by centrifugation. Each pellet was resuspended in 1 ml of the appropriate dilution of concentrated PIC suspensions diluted in tissue culture media without serum to yield multiplicities of infection (moi) of 1.0, 0.1, or 0.01. Control cultures were exposed to diluent alone. The infection was carried out for 1 hr at 37°C with shaking to ensure mixing of cells and virus. Cells were pelleted, inocula were removed and macrophages were resuspended in complete RPMI 1640 or media containing 10 μg/ml *E. coli* LPS. To quantitate apparent cytopathic effect, adherent cells were counted at 24 hr intervals post-inoculation. Cells were counted in 3 separate medium power fields (20X) in duplicate wells using a Nikon inverted tissue culture microscope; cell counts were adjusted to numbers per mm^2 using a stage micrometer, and are reported as mean of the six counts with attendant standard deviations. Macrophage supernatants were harvested at 6 hr intervals post-inoculation for TNF and virus quantitations. Serial dilutions of infected macrophages were cultured in 24 well plates for infectious center assays as described above in order to estimate the efficiency of *in vitro* infection.

Immunofluorescence Assay (IFA): Antiserum against adPIC raised in Hartley guinea pigs was used in IFA to detect viral antigen in cultured macrophages. Acetone or paraformaldehyde fixed coverslips were pre-incubated in 2% normal human serum in phosphate buffered saline (PBS) to block Fc receptors. Slides were then incubated with 1:100-

1:500 dilution of guinea pig anti-adPIC antiserum in PBS for 30 mins, washed extensively, and incubated in FITC goat anti-guinea pig immunoglobulin G (Kierkegaard-Perry) for 30 mins. After washing, slides or cover slips were counterstained with Evan's blue and examined under epifluorescent illumination. Control sections were treated with normal guinea pig serum in lieu of the first step antibody. To detect macrophages, a commercial mouse monoclonal antibody specific for guinea pig tissue macrophages (MR-1, Bioproducts for Science) was used in the above procedure as the first step, with normal mouse serum as a first step control.

Tumor Necrosis Factor Assay: Macrophage supernatants or organ homogenates prepared as 1:10 (w:v) suspensions in tissue culture media were assayed for TNF as previously described (Flick,1984). Spleen homogenates stored at -70°C were thawed once, and clarified by centrifugation at 2000xg for 5 mins prior to assaying. Briefly, L929 cells were distributed in 96 well plates at a concentration of $4x10^4$ per well. After overnight incubation to establish monolayers, the media was removed and replaced with the desired dilution (in general, serial 2-fold increments) of sample to be assayed, or recombinant murine TNFα standard (Genzyme). Actinomycin D (Sigma) was added to each well at a final concentration of 2.5 μg/ml. Plates were placed in a 37°C incubator for 18-24 hrs, then washed 3 times with warmed media, fixed in 10% formalin, and stained with crystal violet. The stain was extracted with absolute methanol, and absorbance was determined at 540 nm on an elisa plate reader (Biotek instruments). Control wells were treated with tissue culture media and 2.5 μg/ml actinomycin D only. Each specimen and dilution was assayed in duplicate. The percent of cytotoxicity for each sample was calculated as shown below:

$$100 \times [1-(\text{mean } OD_{540} \text{ sample wells}/\text{mean } OD_{540} \text{ control wells})]$$

The dilution yielding 50% cytotoxicity was derived from a plot of % cytotoxicity vs. $\log_2$ dilution of sample, and reported as units of TNF after correction for dilution factors in the assay. Where indicated, units were converted to picograms by comparison with the recombinant murine TNF standard. The ability of various anti-TNF reagents (see results) to neutralize TNF was determined by pre-incubating standard recombinant murine TNF (Genzyme), recombinant human TNF (National Institute for Biological Standards and Control, Hertfordshire, England), or supernatants from LPS-stimulated GP peritoneal macrophages with dilutions of test antiserum at 37°C for 60 mins. Samples were then transferred to L929 cell monolayers and assayed as above for residual TNF activity. Immunoadsorption of GP TNF from supernatants was performed using immunoaffinity columns linked with anti-human TNF (Endogen). These experiments were done according to manufacturer's recommendations and as described in figure legends.

Prostaglandin E_2 Assay: Macrophage supernatants were graciously assayed for PGE_2 by Dr. Rodney Baker, Dept. of Pharmacology, Univ. of Colorado Health Sciences Center. The method was enzyme-linked immunosorbent assay (ELISA) (AIA Reagents, Aurora, CO).

Purification of GP TNF and Immunization of Rabbits: In collaboration with Drs. G.A.S. Ansari and M. F. Khan of our Department, we have successfully purified a small amount of guinea pig tumor necrosis factor α. The scheme used was a modification of that described by Tamatani et al. (1989). Briefly, $2x10^8$ resident peritoneal macrophages from 7 outbred guinea pigs were cultured at $1x10^6$ macrophages per ml in RPMI containing 1% (v:v) Nutridoma-SP (Boehringer-Manheim), 2mM L-glutamine, $2x10^{-5}$ M 2-mercaptoethanol, 30 μg/ml gentamicin and 10 μg/ml E. coli LPS. After 48 hrs, culture fluids were

harvested, and cells were removed by centrifugation. The supernatant was sequentially concentrated in an Amicon stirred cell with a YM-10 filter and a Centricon microconcentrator (Amicon) (steps 2 and 4, Table 5). These concentration steps resulted in approximately 330-fold decrease in volume without significant loss of TNF activity. The apparent changes in total protein likely reflect the loss during ultrafiltration of low molecular weight peptides and amino acids which would have been detected in the BCA protein assay (Pierce). The concentrated material was centrifuged at 10,000xg through a millipore .22 micron filter to remove particulate material (step 5). This step resulted in some loss of TNF activity and protein, likely due to removal of residual cell debris and flocculent protein precipitates, possibly containing membrane associated TNF. An aliquot of this clarified concentrated material equivalent to 1 mg of protein was then applied onto a protein C_4 reverse-phase HPLC column (0.46x25 cm.) (Vydac) equilibrated with 10% acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA). The starting buffer (A) was 10% ACN in 0.1% TFA, and the limiting buffer (B) was 90% ACN in 0.1% TFA. The protein was eluted according to the following program: solvent A was run for 10 mins and then a gradient was developed to reach 50% of solvent B in 40 mins. After 51 mins, a second gradient was begun from 50% to 100% of solvent B in 20 mins. The column flow rate was 1.0 ml/min. The absorbance was monitored at 215 nm. Fractions of 1 ml were collected in siliconized tubes, and solvent was removed under vacuum. Fractions were then reconstituted in phosphate buffered saline, and assayed for TNF according to the standard L929 cytotoxicity procedure. Profiles of absorbance at 215 nm and TNF bioactivity in the resulting fractions are shown in Figure 10. TNF eluted at fraction 45-55, consistently running behind an albumin standard. Virtually all of the applied TNF activity and 3.4% of the applied protein were recovered in this peak. These fractions were pooled, and represent 34.3 μg of protein with TNF specific activity of 2.2×10^6 U/mg. Two 2 kg female New Zealand White Rabbits were immunized with 15 μg purified GP TNF as follows: 15 μg of TNF was dotted on nitrocellulose strips, air-dried, then the strips were smeared with complete Freund's adjuvant before the membranes were implanted subcutaneously. One rabbit succumbed to pneumonia; the surviving rabbit was boosted subcutaneously 3 weeks later with concentrated, crude TNF-containing GP macrophage supernatants (approx. 100 μg protein) in incomplete Freund's adjuvant, and re-boosted 8 weeks after initial immunization with 5.5×10^{12} Units of TNF as concentrated GP macrophage supernatants in incomplete Freund's adjuvant. Serum from test bleeds were screened for neutralizing activity as described above, except that the TNF sources were GP macrophage supernatants, or serum from a GP with lethal endotoxemia. Pre-incubation with test serum dilutions was carried out at 4°C overnight before transfer to L929 cells.

Isolation of Lymphocytes: Spleen and lymph nodes were removed using sterile techniques. Blood was collected in heparin at 50 units/ml, and then diluted in an equal volume of HBSS. Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation of the diluted blood through Ficoll-Hypaque (Pharmacia, Piscataway, NJ) at 450xg for 30 mins at room temperature. Splenic lymphocytes were obtained by gentle dissociation of spleens into single cell suspensions. All cells were washed three times in cold HBSS without calcium or magnesium supplemented with 30 μg/ml gentamicin sulfate, and then resuspended in supplemented RPMI 1640. After the final wash, cells were counted with a model ZM Coulter Counter (Coulter, Luton Beds., England) and adjusted to the appropriate cell concentration in supplemented RPMI 1640 containing 5% FBS or, in some experiments, 1% normal guinea pig serum.

Lymphocyte Proliferation Assay: Proliferation assays were performed using $2.5x10^5$ splenocytes per well in flat-bottomed 96 well plates or $1x10^5$ PBMC's per well in round-bottomed 96 well plates in the presence of various concentrations of mitogen. The mitogens used were concanavalin A (Con A) (Calbiochem, San Diego, CA) and phytohemagglutinin (PHA) (Connaught Laboratories Limited, Willowdale, Ontario, Canada). For virus antigen-specific proliferation, spleen cells were placed in RPMI containing 1% normal guinea pig serum and stimulated with dilutions of UV-killed adPIC suspensions concentrated from tissue culture fluids by PEG precipitations as described above. PEG precipitated conditioned Vero cell media was used as "control antigen". Cells were cultured in a final volume of 200 μl supplemented RPMI 1640 at 37°C in a humidified 5% CO_2 atmosphere for the indicated times. Proliferative responses were established by determining the incorporation of tritiated thymidine (^{3}H-Tdr) (ICN Radiochemical, Irvine, CA). ^{3}H-Tdr (0.5 μCi per well) was added to the cultures during the last 18-20 hrs of incubation. Plates were harvested (PhD cell harvester), and incorporation of ^{3}H-Tdr was determined by liquid scintillation counting.

Isolation of Macrophages and T-lymphocytes for Lymphocyte Proliferation Experiments: Macrophages were isolated from single cell suspensions of splenocytes by adherence to plastic in 24 well tissue culture plates. Spleen cell suspensions ($2.5x10^6$ cells/ml/well) in supplemented RPMI 1640, were allowed to incubate overnight at 37°C, 5% CO_2. Nonadherent cells were then gently removed by washing the wells three times in warm HBSS with calcium and magnesium. Plates containing adherent cells were then irradiated with 3,000 rads to prevent any remaining nonadherent cells from proliferating in response to treatment with mitogen.

T-lymphocytes were isolated by adherence to nylon wool, as previously described (Mishell, 1980). Briefly, splenocytes were depleted of adherent cells as described above and adjusted to $5x10^7$ cells/ml in warm, supplemented RPMI 1640. Suspensions of splenocytes were then incubated on a prepared nylon wool column at 37°C for 45 mins. Nonadherent cells were collected by eluting the column at 1 drop per second, and rinsing with an appropriate amount of supplemented RPMI. Adherent column cells were collected by using a sterile syringe to force 50 mls of cold supplemented RPMI 1640 through the column. T-lymphocyte stimulation was performed by adding 1 ml of the T-lymphocyte suspension at $2.5x10^6$ cells/ml to individual wells containing either adherent macrophages or media only. Con A was then added to the cultures, in order to achieve a final concentration of 10 μg/ml of Con A in the cultures. Cultures were incubated for 72 hrs and then proliferation was determined as described above.

Determination of Infection of Lymphocytes: To determine whether isolated T-lymphocytes harbored infectious PIC, splenic T-lymphocytes, macrophages and column adherent cells were isolated as described above, adjusted to 10^6/ml, and then frozen at -70°C and thawed once. Quantities of PIC in these supernatants were determined using the Vero cell plaque assay as described above.

Probes: Uniformly labelled antisense TNFα probe was generated from PstI linearized PGEM3 plasmid containing the murine TNF cDNA (generously provided by Bruce Beutler). This cDNA includes the entire coding region of the murine TNF gene, but lacks the AT-rich repeating octamers in the 3' untranslated region which are common to many cytokines (Caput, 1986). Labelled antisense single stranded RNA probe was transcribed in the presence of ^{32}P-UTP using T7 RNA polymerase according to manufacturer's recommendation (Promega). Specific activities of the probes were greater than 10^9

cpm/μg. The ß-actin probe was generated in a similar manner using T3 polymerase and PstI linearized plasmid template pA1 which contains 1 kb portion of the chicken ß-actin cDNA (Cleveland, 1980). This probe hybridizes with actin genes from a variety of diverse species.

Northern Blot and Slot Blot: Total cellular RNA was isolated from organs or cultured cells using the RNA zol B method according to manufacturer's instructions (Cinna-Biotecx). The amount of RNA in each sample was determined spectrophotometrically. 50 μg of each RNA sample was denatured in loading buffer containing 2.2 M formaldehyde and 50% deionized formamide, separated by formaldehyde agarose gel electrophoresis as described (Sambrook, 1989), and transferred to nylon membrane (Zetabind, Cuno Labs, CT) in 1X SSC buffer. After transfer, the membrane was quickly rinsed in 2X SSC, RNA was cross-linked to the filter by baking at 68°C for 2 hrs. Pre-hybridization was in 0.5M sodium phosphate, 1mM EDTA, 1% BSA, 7% SDS at 65°C for 2 hrs. Blots were hybridized with high specific activity ^{32}P-labeled riboprobes at 50-80°C in 0.5M sodium phosphate, 1mM EDTA, 1% BSA, 7% SDS, 30% formamide. Stringency conditions were titrated for hybridization of murine probes to GP mRNA; optimal specific hybridization of probe to GP TNF mRNA was achieved in 30% formamide at 68°C for 18 hrs. Membranes were washed 3 times at 65°C in wash buffer (40mM sodium phosphate, 1mM EDTA, 1% SDS, pH 7.2), then exposed to Kodak XAR5 X-ray film using intensifying screens at -80°C.

For slot blots, 50 μg of total cellular RNA was dissolved in 1XSSC, 50% formamide, 7% formaldehyde and heated for 5 mins at 70°C. Serial 2-fold dilutions of each sample were prepared in the above diluent, and transferred to Zetabind nylon filters in a vacuum slot blot filtering apparatus. After samples were applied, filter slots were washed 3 times with 1X SSC buffer. Filters were air-dried and baked at 68°C for 1 hr. Blots were pre-hybridized and hybridized as above, washed and exposed to film. Blots were stripped by simmering membranes in 0.1X SSC with 1% SDS for 15 mins and re-hybridized with ß-actin probe under similar conditions. Slot blots were analyzed on a Hoefer GS300 scanning densitometer with accompanying GS360 scanning densitometry software. For each RNA sample, computer integrated "area" for the dilution series of peaks was plotted against $\log_2$ dilution; the dilution yielding a standard reference "area" was derived by linear regression analysis. This reference dilution was used to compare relative amounts of mRNA between specimens. Values were normalized for actin message content by dividing the "relative amount of TNF mRNA" (above) by the similarly derived "relative amount of actin mRNA". Although tissues may differ in terms of actin message on a per cell or per gram basis, this ratio at least serves as a way to standardize for RNA content and compare RNA specimens from a given tissue after different treatments. Hence, results are reported as the degree of change in TNF message levels after *in vivo* treatment with LPS or adPIC.

Western Immunoblot: Pichinde virus antigens were separated by standard SDS-PAGE methods (Laemmli, 1970) using 8% polyacrylamide gels with a 3.5% acrylamide stacking gel. Peptide bands were electrotransferred to nitrocellulose using a Hoefer SemiPhor Semi-Dry transfer unit. Blots were blocked in 5% nonfat dry milk (NFDM) in 50mM Tris-HCl pH 7.6, 0.2mM EDTA, 150 mM NaCl, 0.1% NP40 (TNE-NP40), then exposed to 1:50 dilution of guinea pig anti-adPIC or anti-PIC3739 antiserum in TNE-NP-40 with 1% NFDM for 2 hrs. After washing 5 times for 15 mins in TNE-NP40, filters were incubated with 1μci ^{125}I-protein A (Amersham) for 1 hr, washed, air dried and exposed to Kodak XAR-5 X-ray film using intensifying screens at -80°C.

Histology: Tissues were fixed in 10% neutral buffered formalin, processed through graded alcohols and xylene, and embedded in paraffin. 5 μ thick hematoxylin and eosin stained section were examined.

Statistics: Results are reported as means with standard deviations. Where indicated, statistical significance was assessed using either the Mann-Whitney U-test or Student's two tailed t-test.

RESULTS

I. MACROPHAGES, MONOKINES AND PATHOGENESIS

Establishing a Biological Reference for Studies of Tumor Necrosis Factor and PIC Pathogenesis: A good deal of effort was spent developing a reference paradigm for comparison with the virulent adapted PIC in pathogenesis studies. In our hands, inbred strain 2 guinea pigs were as susceptible as strain 13 guinea pigs to Pichinde Virus (CoAn4763-P15). There appeared to be a slight lag both in clinical illness (weight loss, FIGURE 1) and development of peak splenic viral titers (not shown) in strain 2 guinea pig when compared with strain 13. Never the less, strain 2 guinea pigs were highly susceptible to infection over a wide range of inoculum doses (FIGURE 2). These findings corroborate those of Jahrling et al. (Jahrling, 1981). In subsequent experiments, we attempted to use outbred Hartley guinea pigs, expecting them to segregate into lethal and non-lethal infection groups, since previous published data suggested 50% mortality rate (Jahrling, 1981). However, in two experiments, 2000-3000 pfu of adPIC inoculated intraperitoneally was uniformly lethal in outbred guinea pigs. Results in two separate experiments are summarized in narrative form below; results for one experiment are summarized in tabular form (TABLE 1).

In the first experiment, nine 350 gram female Hartley guinea pigs were inoculated i.p. with 2000 pfu adPIC, and small amounts of blood were removed by cardiac puncture on days 0,3,6,9, and 12, and analyzed for TNF and viral titers. All animals in this experiment succumbed either to apparent effects of viral infection or cardiac tamponade as a complication of the bleeding procedure. (Notably, after animals became febrile about day 6, they demonstrated some bleeding tendency, possibly enhancing the morbidity of cardiac puncture). The mean day of death was day 10; mean log pfu/ml of blood at the time of death (n=6) was 5.96; the mean log pfu/g of spleen at the time of death (n=5) was 8.21. One animal was sacrificed on day 12 because of rectal prolapse. In the second experiment, twelve 800 gram male hartley guinea pigs were inoculated with 3000 pfu of adPIC i.p. and monitored for fever and weight loss. We omitted periodic bleedings and elected to sacrifice moribund animals (defined as 75% of original body weight with subnormal body temperature) along with healthier paired controls, and assay serum and spleen samples for TNF and viral levels. Again, we noted 100% lethality among this group, with the mean day of death at day 12. An unusual hind limb paralytic syndrome developed terminally in many animals. The mean log plaque titer in spleen at the time of death (n=5) was 7.97. Histopathologic findings included lung and liver lesions consistent with PIC infection, as well as an acute, diffuse enteritis of unknown etiology, and mild inflammation in spinal cord gray matter, also of unclear origin. Serologies for LCMV, Sendai virus, SV5 and Reovirus 3 were negative; the sole positive antibody titer in this panel was for Pneumonia Virus of Mice. Of note, spleen cultures were positive for various gram negative "opportunistic" organisms (**E. coli, Proteus mirabilis, Pseudomonas aeruginosa**), suggesting that sepsis contributed to the pathology. The significance of these findings is not known, but we remain concerned about the possibility of 1) superimposed secondary or re-activated latent infection due to PIC induced immunosuppression or 2) emergence of a more virulent reassortment or other mutant viral strain in our stock spleen-passage PIC. We concluded that this approach in outbred animals would not be fruitful, as there was no clear early segregation of animals into a group likely to survive and a group likely to die.

Finally, we abandoned the search for a resistant host in favor of the search for an avirulent PIC strain. This approach utilized PIC CoAn3739, the prototype PIC strain. PIC3739 and PIC CoAn4763 (the parental strain of adPIC) are serologically related based on cross-reactivity in complement fixation assays and lack of significant cross-reactivity with other Tacaribe group arenaviruses by the same method (Trapido, 1971). Partial sequence analysis of the genomes of PIC3739 and the virulent derivative of 4763 (adPIC) indicate that the two strains have similar sequences (10% substitution frequency) for the first 120 nucleotides of the S RNA segment and the first 50 nucleotides of the L RNA segment (Auperin, 1982). Additional comparative sequence information is not, to our knowledge, available at present. We found these two related PIC strains have different virulence properties in strain 13 guinea pigs.

In a pilot dose-response experiment, all guinea pigs inoculated subcutaneously over a 4 log range of PIC3739 doses developed fever of short duration, manifested less than 15% weight loss, and recovered within 12 days post-inoculation (TABLE 2). Intraperitoneal doses of 3000 pfu were chosen in all subsequent experiments for consistency with previous experiments using adPIC. FIGURE 3 shows cumulative mortality for PIC3739 compared to adPIC under these conditions.

Documenting Macrophage Infection <u>In Vivo</u>: Initially in this model, we sought to confirm previous immunofluorescence data suggesting that macrophages are a major target of infection by PIC. Focus forming assays were performed on a variety of organ macrophages to demonstrate productive infection of macrophages during experimental PIC infection. Spurious cytotoxic effects of macrophages products on Vero cells in this assay were ruled out by confirming the presence of viral antigen in cytolytic plaques by transferring plaques to nitrocellulose filters and probing these filters with antiserum specific for PIC antigens, as described in Materials and Methods. FIGURE 4 shows a representative experiment. A higher proportion of peritoneal and splenic macrophages from adPIC infected GP's harbored infectious virus than those from PIC3739 infected animals. There appeared to be a decrease in the percentages of macrophages infected during the second week of experimental infection. Whether this is the result of putative viral killing of infected cells, successful clearance of virus, or some artifact of the system, is not known. The proportion of infected splenic macrophages was consistently lower than that of peritoneal macrophages, perhaps in keeping with the intraperitoneal route of infection. Alveolar macrophages and liver sinusoidal lining cells (including Kupffer cells) were also productively infected to a comparable degree (data not shown). Examination of explanted adherent peritoneal macrophages for adPIC viral antigen by IFA yielded parallel but slightly higher figures; between 5% and 18% of macrophages showed specific fluorescence, with roughly half this number (2.5%-10%) expressing viral antigens on the cell surface as judged in paraformaldehyde fixed smears (data not shown). Immunofluorescence of total and adherent peritoneal cell populations indicate that adherent macrophage populations are neither enriched for nor depleted of virally infected cells. The discrepancy between immunostaining and infectious center assays suggests that a proportion of macrophages contain viral antigen (killed virus, defective virus, partially degraded viral proteins?) without being productively infected at these times after infection.

Documenting Tumor Necrosis Factor-Like Bioactivity in Serum and Organ Homogenates During PIC Infection: As a first step in determining whether TNFα plays a role in PIC pathogenesis, we measured TNF levels in serum of guinea pigs infected with adPIC or PIC3739. As shown in TABLE 3, TNF was detectable in the serum of a small, variable

proportion of adPIC infected guinea pigs during the first post-inoculation week. However, late in infection (post-inoculation days 11-15), when adPIC infected guinea pigs were moribund, 30-100% of animals had measurable TNF in their serum. In contrast, none of the 15 guinea pigs infected with the attenuated strain (PIC3739) had measurable TNF at any time during the course. Mean serum TNF levels for adPIC infected guinea pigs are shown graphically in FIGURE 5, demonstrating an apparent rise in serum TNF levels during the course of infection, with a peak in the immediate pre-lethal period.

TNF-like cytotoxic activity was also found in spleen homogenates from guinea pigs infected with either PIC strain (FIGURE 6). A different temporal pattern of TNF expression was seen in spleen than in serum, with levels peaking during the first week of infection, at around the time of onset of fever and before significant weight loss. TNF-bioactivity then declines below control levels. Although FIGURE 6 suggests that PIC3739 incited slightly higher levels of spleen TNF than did adPIC, the numbers of animals used was too small to achieve statistical significance. In any case, spleens from virally infected guinea pigs did generate statistically significant higher levels of TNF than control uninfected spleens. This suggests that early local TNF may serve a protective role, and the later appearance in serum may be a marker of failed local control of virus with spill-over of this potent monokine into the serum.

TNF mRNA Levels in adPIC Infected Guinea Pigs: The identity of this TNF-like cytotoxic material could not be confirmed in neutralization experiments, as effective neutralizing antibodies against GP TNF are not available (see below). Thus, homology of murine and GP TNF at the mRNA level was exploited in performing slot blot analyses to determine whether changes in TNFα gene expression correlated with the TNF-like bioactivity detected in serum and organ samples. Total cellular RNA was extracted from organs of normal, LPS treated, and adPIC infected (post-inoculation day 6) guinea pigs. A murine TNF cDNA probe (generously provided by B. Beutler) was utilized; optimal hybridization conditions were established by Northern blot analysis. Under conditions selected for the slot blot experiment, Northern blots from RAW264.7 cells (a TNFα-producing murine macrophage cell line), and guinea pig tissues demonstrated bands of appropriate size that are consistent with TNF mRNA (data not shown). Relative TNF mRNA values derived from scanning densitometry of slot blots and standardized for ß-actin mRNA levels are shown in TABLE 4. Values are normalized to heart, which was expected to manifest low levels of TNF mRNA. The 2.7-fold increase in TNF mRNA in spleen from adPIC infected guinea pig at day 6 compared to uninfected guinea pig supports the contention that the bioactive material present in spleen homogenates is at least in part TNFα. The cDNA probe will not hybridize to TNFß, a potential confounder in the bioassay (Caput, 1986).

TNF Secretion by Explanted Macrophages: In order to elucidate potential cellular sources of TNF, we assayed supernatants from peritoneal and splenic macrophages explanted at various times during infection. Explanted macrophages which were not further stimulated in culture showed inconsistent, very low levels of "constitutive" TNF production, which were not significantly different from parallel control macrophage cultures (data not shown). More striking was the "priming" effect of adPIC infection on LPS-induced TNF production by PEC from moribund animals (FIGURE 7A). Interestingly, whereas day 11 macrophages showed augmented TNF production to an LPS stimulus, day 6 macrophages were suppressed in their ability to secrete TNF in response to LPS. Similar trends were seen among PIC3739 macrophage explants, although to a lesser degree

and not statistically different from control cultures. In contrast, splenic macrophages showed diminished TNF production to LPS challenge throughout the disease course, a trend seen in both viral strains (FIGURE 7B). This finding is somewhat at odds with evidences of TNFα in spleen homogenates at day 6. Since clarified spleen homogenates were prepared in such a way that cell membranes and fragments are likely present, we suggest that TNF bioactivity in tissue homogenates represents a locally produced membrane associated 26kd form of TNF, rather than the 17kd secreted form (Kriegler, 1988). Alternatively, TNF bioactivity may reflect TNFß produced by splenic lymphocytes. Although we cannot rule this possibility out in the absence of a specific neutralizing antibody for GP TNFα, the up-regulation of specific TNFα mRNA in spleen implies that at least a portion of the bioactive material is TNFα. Taken together, ex vivo macrophage data suggest that peritoneal macrophages are a source for TNF late in experimental infection, whereas, splenic macrophages may produce local cell-associated TNF earlier in the course.

Neutralization of Guinea Pig TNFα: In an effort to verify that the TNF-like bioactivity in serum and spleen was in fact TNFα, we attempted to identify antisera that are capable of neutralizing guinea pig TNFα. Using a commercial rabbit anti-murine TNFα antiserum (Genzyme), or rabbit anti-human TNFα antiserum (Endogen) in solution, we were able to abrogate about 70% and 40% of cytotoxic activity in LPS-activated guinea pig macrophage supernatants respectively (FIGURES 8 and 9). Immuno-adsorption of guinea pig macrophage supernatants against commercial anti-human TNF immunogel (Endogen) removed less than 50% of cytotoxic activity, under conditions that neutralized activity in a purified human TNFα sample (data not shown). These observations of the relatively poor neutralizing ability of antisera raised against TNF of heterologous species is supported in the literature (Sheehan, 1989). This led us to purify guinea pig TNFα by HPLC in order to raise our own neutralizing antiserum. 30 μg of TNFα were isolated (FIGURE 10), representing a 917-fold purification over starting supernatant (TABLE 5). Two rabbits were immunized as described in Materials and Methods; one rabbit died due to pneumonia. At the time of this report, the surviving rabbit had been immunized with 15 μg of purified GP TNF, then boosted twice with concentrated GP macrophage supernatants. Thus far, this rabbit has generated an insignificant level of neutralizing antibody [approximately 33% neutralization was the maximum achieved with this reagent (data not shown)]. Thus, we have not yet succeeded in developing an antiserum with sufficient neutralizing titer to confirm the presence of TNFα in guinea pig samples.

Other Monokines in Experimental PIC Infection, In Vivo: We were interested in examining the pattern of secretions of other monokines during adPIC infection. In particular, prostaglandin E_2 was of interest in light of its immunosuppressive properties. In two experiments, prostaglandin E_2 (PGE_2) was assayed in supernatants from peritoneal macrophages explanted during adPIC infection. (These same supernatants were also assayed for TNFα). Although statistical significance is not achieved in this small sample, data in FIGURES 11A and 11B suggest that this immunosuppressive monokine is secreted in higher levels in both unstimulated and LPS-treated macrophages from day 6 adPIC animals compared to controls. PGE_2 is known to decrease TNF production by macrophages (Scales, 1989); it is tempting to theorize that this observation accounts in part for apparent suppression of LPS-induced TNF production in macrophages from day 6 adPIC infected guinea pigs. These observations have not yet been extended to PIC3739 infected macrophages.

Comparison of adPIC and PIC3739 Infections In Vivo Miscellaneous Pathology: Having shown that these two virus strains diverge in terms of lethality and induction of serum TNF, we sought to characterize other comparative aspects of the host-virus system. adPIC consistently achieved plaque titers of 1-2 logs higher in blood and spleen than did PIC3739 (FIGURES 12A and 12B). This was seen even at day 3 post-infection, suggesting either an inherent replication advantage in adPIC or increased efficiency of natural immunity against the attenuated strain PIC3739.

Significant changes in peritoneal cell populations were noted after i.p. infection with either strain. At day 3, there was a mild increase in peritoneal macrophage (PM) numbers, without significant lymphocytosis by day six, there was a slight decrease in absolute PM numbers in both viral strains, and a pronounced accompanying lymphocytosis. By day 11, PM yields were drastically reduced in adPIC infection, and slightly elevated in PIC3739 infection (FIGURE 13). Both cases were associated with persistent lymphocytosis (20-40%). PIC3739 animals sacrificed at day 15 showed a similar trend in terms of decreasing PM numbers. Interestingly, if one groups animals according to moribund vs. recovering status irrespective of infecting virus (see below), very low PM numbers were significantly associated with clinical disease. The central question remains as to whether this decrease in the number of recoverable PM is due to frank killing of these cells by virus or altered kinetics of macrophage recruitment to and egress from this site.

As noted above, there was about a 10% mortality rate among inbred guinea pigs inoculated intraperitoneally with 3000 pfu of PIC3739, as opposed to those receiving a subcutaneous dose. Certain features were associated with mortality, no matter which virus strain was introduced. Consistent findings in this group (irrespective of which virus was given) included marked decrease in recoverable peritoneal macrophage numbers (see above), and absence of splenomegaly. Histologically, this group of animals displayed deposition of fibrinoid material in the marginal zones and red pulp of the spleen, with some granularity and collapse of architecture in the red pulp. By contrast, animals who showed evidence of recovering or surviving the infection had significantly enlarged spleens with expansion of the red pulp (macrophage rich zone) and minimal fibrinoid change. There was no white pulp hyperplasia or necrosis is either group. A second histologic feature peculiar to the moribund group was pronounced infiltration of the lamina propria of the gut by foamy macrophages containing cytoplastic debris. (This finding was also remarked upon by Dr. Jensen and colleagues at our July 1990 meeting, where they speculated upon the role of a maladsorptive syndrome on wasting disease in PIC infected guinea pigs. We now share these speculations with the G.W. group). TABLE 6 outlines other histopathologic findings; hepatic inflammation, steatosis, lymph node sinus histiocytosis, pneumonitis etc., appeared to be distributed evenly among the two groups and did not correlate with outcome. The acute neutrophilic enteritis seen in the outbred guinea pigs was not observed in this group. Additionally, bacterial cultures of spleen tissue from all animals in the moribund group were negative. Thus, a suggested association between lethality and superimposed infection with opportunistic coliforms is not confirmed in this experiment.

In Vitro Infection of Peritoneal Macrophages with PIC: In vitro infection of peritoneal macrophages with adPIC and PIC3739 were performed in order to compare permissiveness of macrophages for infection with the two viral strains, and their abilities to influence TNF production. In general, at a given multiplicity of

infection, adPIC infected macrophages produced more infectious virus than did PIC3739 infected macrophages (FIGURE 14). As determined by focus forming assay, adPIC demonstrated a slightly higher efficiency of establishing infection in the target cells (data not shown); however, this difference did not appear to account for the entire difference in viral yields during the 72 hr experiment. Both of these in vitro observations closely paralleled findings in vivo. There was no simple relationship between viral infection and TNF production (FIGURES 14 AND 15). However, the following patterns emerge: 1) in unstimulated macrophages, TNF production was seen only in adPIC infection at the highest MOI. This low level of TNF production was associated with a slow rate of increase in virus release. Parallel cultures with steeper rates of virus elaboration showed no measurable TNF in their supernatants. 2) In LPS-stimulated macrophages, suppression of LPS-induced TNF production was seen at two ends of the range of "viral burden" (if we equate viral burden with levels of infectious virus produced). Thus, dampening of the LPS response was seen at very high viral burdens (adPIC 1.0), and at lower burdens. This suppression tended to be associated with increasing levels of virus production. 3) Augmentation of LPS induced TNF production was seen at intermediate viral burdens, and was associated with decreasing virus levels (as cause or effect?). A biphasic relationship between post-infection interval (and/or viral burden) and TNF production is suggested by these data; i.e., there may be an "early" decrease in inducible TNF production at low virus levels, a transient priming effect on TNF production as virus levels reach a certain threshold, followed again by dampened TNF response to LPS when virus replication is very brisk. Such a pattern may also fit the ex vivo data, where suppression of LPS induced TNF was seen at day 6 for PEC, while augmentation was seen at day 11 in adPIC infection. Alterations in TNF secretion may be mediated by other monokines; notably interferons, TGFß, and prostaglandins can suppress TNF production by macrophages in an autocrine and paracrine fashion. Data is not yet available on the potential roles of such monokines in this system.

LPS activated macrophages were clearly capable of limiting virus production. The data in FIGURE 16 summarized above suggests a possible role for TNF in viral killing, or at least an association between TNF production and reduction in viral yield.

The central question of whether either virus strain can overtly kill macrophages it infects has not been satisfactorily answered. By direct manual counting of adherent macrophages after in vitro infection, no statistically significant difference in viability between PIC infected and uninfected control cultures was found in several experiments. However, this method is insensitive due to the uneven distribution of macrophages in wells and the potential for sampling errors in fields counted. Other methods, including trypan blue exclusion are not suitable because of the phagocytic nature of these cells. The MTT assay for cellular dehydrogenases (Tada, 1986) was attempted; however, "activated" macrophages were found to reduce the substrate to a higher degree than unstimulated macrophages, confounding the results.

II. LYMPHOCYTES, IMMUNOSUPPRESSION AND IMMUNITY

Cross-Protection of Guinea Pigs Against adPIC Infection by Immunization with PIC3739, and Lymphocyte Proliferation to Heterologous PIC Antigens: Guinea pigs infected with PIC3739 over a 4 log range of doses were protected against challenge with 10^4 pfu adPIC (TABLE 7).

Spleen cells from immunized and challenged animals were tested for lymphoproliferative responses to adPIC antigen. Results from these two preliminary experiments are shown in FIGURES 17 and 18. They indicate that:

a) we are able to elicit PIC specific lymphoproliferative response with secondary or tertiary exposure to viral antigens in vitro. Spleen cells sensitized to PIC3739 recognized and proliferated to adPIC antigen.

b) splenocyte proliferative responses to T cell mitogens were normal in guinea pigs weeks after immunization with avirulent strain and challenge with the virulent strain (data not shown).

c) there was a suggestion that in vivo challenge after immunization resulted in a decrease in antigen specific lymphocyte proliferation (LP) compared to immunization with avirulent strain only. The effect was most prominent at the longer post-challenge interval (expt. #2). This observation defies the expected "booster" effect of challenge with this related virus. However, LP responses of animals infected and subsequently challenged with the parental strain need to be compared with those for animals challenged with adapted strain as above.

Pichinde Virus Antigens: Indirect immunofluorescence (IFA) assays on sera from this group of animals showed a somewhat similar pattern in humoral responses; i.e., there was no "boost" in antibody titers after challenge with adapted strain (TABLE 8). There was significant cross-reactivity between antigens of the two strains, as expected; guinea pigs exposed to either adPIC or PIC3739 alone developed cross-reacting antibodies against the heterologous strain.

Western immunoblots confirmed antigenic cross-reactivity and also showed distinct antigenic differences between adPIC and PIC3739. Antiserum raised against PIC3739 recognized adPIC antigens of approximately 70 and 63 kilodaltons. The analogous immunoblot of PIC3739 displayed a tightly spaced doublet at 69-72 kd, and a faint band at 31 kd (FIGURE 19). These results are a promising indication that differing pathogenesis phenotypes of these two poorly characterized virus strains may correlate with structural differences.

Demonstration of Diminished Responses of Splenocytes and Peripheral Blood Mononuclear Cells (PBMC's) to Mitogenic Stimulation: Cell mediated immunity is presumed to be of greater importance than humoral immunity in the resolution of arenavirus infections (Johnson, 1987). To explore the possibility that such immune responses are impaired in guinea pigs infected with the virulent adPIC virus, we examined the in vitro T-lymphocyte mitogen responses of lymphocytes isolated from animals at different times after adPIC inoculation. (Similar experiments have not yet been done using the PIC3739 strain). Diminished responsiveness of lymphocytes to mitogenic stimulation in vitro,

is often indicative of in vivo generalized immunosuppression (Liener, 1986). We examined the in vitro responses of lymphocytes isolated from animals at different times after adPIC inoculation to T-lymphocyte mitogens. Depression of splenocyte proliferative responses to Con A was seen at days 3, 7, and 11 after inoculation (FIGURE 20). Responses to optimal and suboptimal Con A concentrations of splenocytes isolated from animals 3 days after adPIC inoculation were inhibited 10-50% depending on the animal or concentration of mitogen examined (FIGURE 20A). Statistical analyses indicated that most of these decreases at day 3 were statistically significant. By post-inoculation days 7 and 11, dramatic decreases (80-95%) in the splenocyte proliferative response to optimal and suboptimal concentrations of Con A were observed (FIGURES 20B and 20C). Splenocyte responses to PHA or supraoptimal concentrations of Con A were similarly impaired (data not shown). Additionally, dramatic decreases in PBMC responses to Con A and PHA 7 days after infection were observed (FIGURE 21). Decreases in responses to Con A of PBMC's isolated from infected animals 11 days after inoculation were also observed in preliminary experiments (FIGURE 22). All of the decreases present 7 and 11 days after inoculation were statistically significant.

Splenocytes From Infected Animals do not Demonstrate Delayed Kinetics of Response to Mitogenic Stimulation: To rule out the possibility that the above data reflects simply a delay in the responses of splenocytes infected animals to mitogenic stimulation, spleen cells from day 11 post-inoculation animals were cultured for prolonged periods (72, 96, and 120 hrs) before measuring thymidine uptake. Spleen cells from infected and uninfected control animals showed similar kinetics of proliferation to this mitogen, and there was a statistically significant depression in ^{3}HTdR uptake by infected spleen cells at all time points (FIGURE 23).

The Defect(s) in Mitogenic Responses of Splenocytes is(are) Associated with the Lymphocyte and not with the Macrophage: Macrophages can act as accessory cells for the induction of T-lymphocyte proliferation in response to stimulation with mitogen. It is possible that infection of animals with adPIC may affect the function of splenic macrophages, T-lymphocytes, or both. To discern which of these was the case, lymphocytes and macrophages were isolated from spleens of adPIC-infected and control animals 11 days after inoculation. Lymphocytes isolated from infected animals were examined for their ability to respond to mitogen in the presence of macrophages from control animals. Additionally, lymphocytes isolated from control animals were tested for their ability to respond to mitogen in the presence of macrophages from infected animals. In these experiments macrophages from infected or control animals supported normal proliferative responses of lymphocytes from control animals; however, lymphocytes from control animals displayed a proliferative response to mitogen more than 100-fold greater than the response of lymphocytes from infected animals in the presence of macrophages isolated from either control or infected animals (TABLE 9). These data are consistent with the hypothesis that adPIC infection alters T-lymphocyte function, but not the ability of macrophages to support mitogen induced proliferation of T cells.

Nylon Wool-Nonadherent T-lymphocytes from Infected Animals Contain Very Low Amounts of Plaque Forming Pichinde Virus: It is possible that lytic infection of T cells by adPIC accounts for the apparent decrease in mitogen responsiveness of unfractionated spleen cells populations. However, in several experiments, there were

no gross differences in the percentages of recovered nylon-wool non-adherent cells from spleens of infected vs. uninfected animals; in each case, 25-40% of spleen cells previously depleted of plastic-adherent cells passed through nylon wool (data not shown). Plaque assays of freeze-thawed spleen cell fractions from post-inoculation day 10 guinea pigs yielded 3000-6000 pfu of virus per 10^6 nylon wool passed cells, suggesting that a maximum proportion of 0.3-0.6% nylon-wool passed cells are productively infected. This T cell enriched population was likewise twice depleted of macrophages by plastic adherence followed by nylon wool adherence. However, even a small percentage of virally infected contaminating cell populations could easily account for the small amounts of virus detected in this assay. These preliminary results suggest that T cells are infected at a low rate compared to macrophages, and that selective viral lysis of T cells is unlikely to account for depressed mitogen responses seen late in infection.

DISCUSSION

Our two-pronged research effort using the PIC model of arenavirus disease has yielded significant results along 2 fronts; namely 1) the role of macrophage infection and monokine production in disease pathogenesis, and 2) mechanisms of immunity and the role of immunosuppression. In the second year of the contract, new data has arisen concerning the comparison of two (poorly characterized) PIC strains of differing virulence for guinea pigs. Use of the avirulent PIC3739 strain has indicated that the presence of TNF in serum is associated with lethal outcome, strengthening the hypothesis that TNFα plays a role in pathogenesis of arenavirus disease in guinea pigs. Additionally, cross-protection studies and nascent evidence of antigenic differences suggest PIC3739 as putative vaccine strain model for arenavirus disease.

Virulence in this PIC strain pair model is associated with higher levels of viral replication in organs and blood. This is clearly reminiscent of human Lassa Fever, in which level of viremia is a predictor of outcome (Johnson, 1987). We have shown that this in vivo phenomenon is paralleled in in vitro infection of macrophages, i.e., the virulent adPIC strain replicates more rapidly in macrophages than does the avirulent strain. The reproducible 10-100-fold differences in gross total virus yields after in vitro infection is not entirely explained by some advantage of the virulent strain in establishing infection, as focus forming assays demonstrate a less than two-fold difference in the proportions of macrophages productively infected in vitro or in vivo by the two PIC strains. This system appears somewhat novel in that quantitative viral kinetics has not often been cited in clinical literature as a virulence factor (Johnson, 1987). The fact remains that, in PIC as in human Lassa Fever, very high virus levels are not reflected in severe pathologic alterations in any organs (Winn, 1975). If not by direct cytopathology on infected cells, how then do the more rapidly replicating arenaviruses exert their fulminant pathologic effects?

It has been suggested by others (Murphy, 1977) and proven by infectious center assays by us that PIC infects cells of the reticuloendothelial system. The issue of whether this is a frankly cytopathic/cytolytic infection remains unresolved, and experimentally difficult to address. Previous work suggested that PIC is not frankly cytopathic for murine macrophages (Friedlander, 1984). We have not to date satisfactorily directly documented cytopathic effect in guinea pig macrophages infected in vitro. However, we have observed apparent redistribution or altered migration patterns in visceral macrophage populations after in vivo infection. Lethal infection is invariably associated with drastically reduced numbers of recoverable peritoneal macrophages. There is a coincident decrease in spleen size, presumably due to depletion or necrosis of macrophage rich zones; expansion of the lamina propria of the small bowel by macrophages is also prominent in animals dying of PIC infection. It has been demonstrated that peritoneal macrophages migrate to the spleen among other visceral sites (Roser, 1970). This traffic may provide a mechanism for viral dissemination to other macrophage rich organs. Does the more rapidly replicating virulent virus somehow alter the kinetics of recruitment or egress of peritoneal macrophages? Does the slower replicating virus foster expansion of splenic red pulp by inflammatory macrophages as part of a successful immune response? These questions are highly speculative. The pattern of visceral macrophage distribution in association with virulence offers tantalizing clues to as yet obscure elements of pathogenesis.

A central hypothesis of our work is that PIC infection may alter secretory function of target macrophages and that pathology is in part monokine-mediated. In this light, it is tempting to attribute pathogenetic significance to the association of serum TNF activity and severe disease in this model. Many of the pathologic and pathophysiologic changes seen in adPIC infected guinea pigs mimic those seen in TNF-mediated endotoxic shock (Zuckerman, 1989). The profound wasting seen in these animals is reminiscent of that produced by chronic low level TNF administration (Tracey, 1988). TNF has been shown to induce hepatic lipogenesis (Feingold, 1987), correlating with the impressive histologic fatty change seen in moribund adPIC infected guinea pigs. TNFß (lymphotoxin) is a T-cell derived lymphokine which shares many biological properties with cachectin/TNFα, including ability to kill L929 cells (Paul, 1988). Although the common pro-inflammatory properties of the two TNF's have been emphasized, only TNFα has been implicated in metabolic and shock phenomena (Paul, 1988). We have not proven that cytotoxic material is in fact the monokine TNFα. Because of the lack of availability of guinea pig specific anti-TNF reagents, we have been limited to the use of the standard bioassay for TNFα determinations. Possible spurious sources of cytotoxicity include direct viral lysis of L929 cells in the assay; we feel we have ruled this out by demonstrating that heating specimens at 60°C for 30 mins [conditions which inactivate virus-(Allison, 1985)] did not significantly change cytotoxic activity. The activity we routinely measure in macrophage supernatants has many features of cachectin--i.e., heat stable, LPS-inducible, and produced by adherent cell populations with no detectable lymphocyte contamination.

The physiologic role of circulating TNF is not clear. TNFα has a very short half life in the serum (Tracey, 1988), and is found only inconstantly in sera in a variety of human infectious and neoplastic diseases (Fong, 1990). Clearly TNF has different functions and pathologic effects depending upon which anatomic "compartment" it is expressed or released in. In a rat model of peritonitis, serum TNF, but not TNF activity in peritoneal fluid, was associated with lethality (Bagby, 1991). Anti-TNF antiserum administered intravenously, but not intraperitoneally, decreased mortality from peritonitis/sepsis. Additionally, a cell-associated form of TNF exists and may play an important in focussing local inflammatory effects of this potent monokine (Kriegler, 1988). In a rat model of burn and sepsis, cell-associated TNF in liver tissue was expressed earlier and more constantly than serum TNF (Keogh, 1990). Our finding of TNF-like activity in spleen homogenates (and a corresponding increase in TNF mRNA levels in spleen) in the first week of adPIC or PIC3739 infection also implies that local production of TNF has an important immunologic role. Our assay conditions of clarified spleen homogenates do not exclude the possibility that we are measuring a cell associated form of TNF. Other investigators have advocated the use of detergents to solubilize membrane associated TNF from organ homogenates (Nakane, 1992) and make such measurements more reproducible. We hypothesize that the expression of soluble and/or cell-associated forms of TNF in the spleen of PIC-infected guinea pigs during the period of most rapid viral replication represents a facet of the physiologic inflammatory, immune response aimed at controlling the infection. In this context, it is interesting that PIC3739 infected animals manifest higher levels of spleen TNF-like activity than adPIC infected animals. In vitro, TNFα demonstrates antiviral effects that synergize with, but are separate from interferon properties (Mestan, 1986 and Wong, 1986). We believe it likely that the slower replicating PIC3739 strain is successfully controlled by the immune response, with TNF possibly playing a role, whereas, adPIC overcomes the immune response, leading to dysregulation of monokine

production. To the extent that the immune response can be separated into "protective" and "pathogenic" arms, the later appearance of TNF in the serum may represent the overflow of this mediator after the failure of local control of viral replication; circulating TNF may then trigger the cytokine cascade associated with wasting and shock in the terminal phases of the disease. Thus, TNF and other cytokines may be viewed in this model both as mediator of the lethal effects of adPIC infection and as marker of an unsuccessful immune response.

The simplistic notion that PIC infection alone induces TNF overproduction proved incorrect; neither macrophages explanted from infected animals, nor macrophage infected at various multiplicities of infection in vitro showed an appreciable increase in "constitutive" TNF production. PIC thus differs from Sendai virus (Aderka, 1986), Influenza A (Nain, 1990), and other viruses capable of infecting macrophages and triggering TNF production. Instead, we observed variable enhancement of LPS-induced TNF production, suggesting that at certain stages of infection, PIC was capable of "priming" macrophages for TNF secretion to a second stimulus. This situation has been well documented for influenza A, and is attributable to both increased transcription and increased stability of TNF mRNA in virus infected, LPS-treated cells (Gong, 1991). We have not to date examined TNF mRNA levels in isolated macrophages. Evaluation of explanted macrophages or macrophages infected with PIC in vitro suggested a complicated relationship between the degree to which a population was infected and TNF production. Furthermore, trends in TNF secretion seen in adPIC infected cells were also seen in PIC3739 infected cells, but with a lag period corresponding with the delay in achieving comparable viral levels. These observations have led us to theorize that PIC effects on TNF secretion depend on some measure of viral "load", and that a biphasic relationship exists between virus replication and TNF elaboration. In particular, suppression of LPS-induced TNF production was seen in peritoneal macrophages explanted from adPIC infected guinea pigs during the first post-inoculation week, and to a lesser degree from PIC3739 infected animals. This was followed by a rebound potentiation of LPS-induced TNF production by day 11 adPIC infected peritoneal macrophages. Similar levels of TNF overproduction to LPS stimulus were seen in day 15 PIC3739 peritoneal macrophage explants. Splenic macrophages from all animals were suppressed in terms of LPS-induced TNF production. Although differing levels of macrophage killing by the two virus strains could not be accurately determined, and TNF levels were not standardized to viable cell numbers at the time of supernatant harvest, we feel that these experiments accurately reflect the capability of a population of infected cells to respond to LPS. If TNF underproduction after LPS stimulus in ex vivo experiments was associated with increased cell killing by virus, then one may surmise that a similar population of macrophages would be similarly unable to respond in vivo for similar reasons. Nonetheless, there is a precedent for virus induced inhibition of TNF production, operative at the transcriptional level very early in infection, in Epstein-Barr virus infected human monocytes and monocytic cell lines (Gosselin, 1991).

These findings in ex vivo cultures have some correlates in preliminary in vitro infection experiments. As described above (Results), these data suggest that inhibition of LPS-induced TNF production occurs in peritoneal macrophage cultures shortly after infection, or when relatively low levels of virus are released and at the opposite end of the spectrum, where virus production is very high. By contrast, in an intermediate range of virus release (irrespective of PIC strain), macrophages manifest "priming" for TNF production after LPS stimulus. Any stimulatory effects of endotoxin

or other substances in the buffer in which concentrated virus inocula were suspended can not be entirely ruled out in this experiment. However, the fact that infected, unstimulated macrophages did not elaborate TNF (reproducing our ex vivo data) suggests that such confounders do not account for the results. We suggest that this patterned TNF response may relate to sequential production of various suppressive monokines in response to PIC infection; some evidence presented here suggests that PGE_2 may play a role in the early TNF suppression. Other cytokines are difficult to measure in the guinea pig system. Shifts in monokine profile with stage of infection have been cited in SIV infection of macaques (Horvath, 1991). These authors showed that alveolar macrophages had different TNF responses to LPS stimulation depending upon whether they were isolated from animals who were SIV seropositive or had clinical AIDS. Responsiveness could not be correlated with productive SIV infection of macrophages, which was undetectable in both populations. Panuska et al. (1990) noted that exposure of human alveolar macrophages to respiratory syncytial virus (RSV) caused increased transcription and secretion of TNF. This phenomenon was clearly dependent on the dose of virus. Therefore, in other virus/macrophage systems, evidence links alterations in monokine (TNF) production with either systemic immune status changes resulting from disseminated infection or features inherent in the direct virus-macrophage interaction. Both principles may certainly be operative here. The ability of adPIC infection to "prime" macrophages for TNF production may be of some importance in light of intestinal lesions that were noted; mucosal breaks, particularly in the large intestine, would increase the amount of "endogenous" LPS absorbed from the gut. Infected guinea pigs with hyper-responsive mononuclear phagocytes may then be more prone to lethal consequences of exposure to LPS, via TNF overproduction. Such a scenario has been proposed in murine graft vs. host disease (Nestel, 1992). In this model, the authors suggest that macrophages are "primed" for TNF production by interferon-γ generated during the afferent limb of the graft vs. host reaction. This priming in combination with gastrointestinal mucosal lesions characteristic of GVHD likewise sets these mice up for lethal endotoxic shock.

We have begun to address fundamental issues of immunity in this model by documenting that adPIC infection is associated with profound immunosuppression in vitro. (Comparisons between the adPIC and PIC3739 have not been done). Although generalized immunosuppression and secondary opportunistic infections are not features of human arenavirus disease (Cummins, 1990), immunosuppression may be of primary pathogenetic significance if arenavirus-immune system interactions impede the development of an effective specific anti-viral immune response. Virus induced immune suppression is well documented in many human and veterinary diseases and experimental systems. Several mechanisms of virus-induced immune suppression have been discussed in the literature; these include direct viral infection of lymphocytes, production of host or virally derived immunosuppressive substances, increased T suppressor cell activity, or interference with various macrophage roles in the immune response (Rouse, 1986). Direct infection of T cells may cause frank cytolysis with induction of lymphopenia, as in parvovirus or HIV-1 infections; alternatively, in non-cytocidal T cell infections, differentiation and appropriate immune functions of T cells may be impaired, as in measles (Rouse, 1986). We have evidence in our adPIC system that hyporesponsiveness of spleen cells to T cell mitogens is not due to loss or destruction of responsive cells with nylon adherence properties of T cells.

Cultures of macrophages derived from infected guinea pigs appeared to produce more adPIC than did cultured nylon wool enriched spleen cells ("T-lymphocytes"). Interestingly, despite the fact that macrophages are an important viral target, macrophages from infected animals were able to support mitogen-induced proliferation of normal guinea pig spleen lymphocytes. Conversely, nylon wool enriched spleen cells (T-lymphocytes) from infected animals were hyporesponsive to mitogen when cultured with macrophages from infected and uninfected animals. Thus, the defect in lymphocyte proliferation of spleen cells from infected animals appears to reside in lymphocytes rather than in macrophages. The implication of a primary T cell defect leaves open the possibilities of mediation of immunosuppression by viral or host derived suppressive factors. For example, the retrovirus feline leukemia virus produces a protein P15(E) which is capable of inhibiting mitogen-induced proliferative responses of normal lymphocytes (Rojko, 1984). Interferons, particularly interferon γ, are known to display potent immunosuppressive effects in vitro (Brenan, 1983; Rouse, 1986; Schattner, 1983). Measles virus also causes lymphocytes to produce a presently unidentified product which significantly reduces the proliferative response of T cells to mitogen (Sanchez-Lanier, 1988).

In summary, we believe that we have established a system suited for coordinated study of cytokine induced pathology and immune dysfunction. Continued investigations of PIC/immune system interactions and better characterization of the two strains with different virulence phenotypes will lend valuable insights into the pathogenesis of a group of poorly understood human hemorrhagic fevers. Futhermore, biochemical and molecular characterization of the two strains of Pichinde virus may provide valuable insights into the components of the virus important for these differences in pathogenicity. We propose that analyses of these two virus strains may provide the basis for the development of attenuated vaccine strains of Lassa Fever using molecular biological approaches. Defining the differences in these two virus strains can then be corrected with PIC/immune system interactions and lead to a better understanding of arenavirus induced disease, its control and treatment.

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FIGURE 1: Guinea Pig Body Weights During adPIC Infection.

500-700 gram strain 13 or strain 2 guinea pigs were inoculated intraperitoneally with 30 to 3000 plaque forming units of adPIC on day 0. A total of 14 strain 2 and 12 strain 13 animals from three separate experiments are represented. All animals of both strains were febrile by day 5-6 post-inoculation. Control animals injected with tissue culture media gained weight and did not develop fever.

Figure 2: Weight Loss and adPIC Dose in Strain 2 GP.

600 gram female strain 2 guinea pigs were inoculated intraperitoneally with the indicated dose of adPIC and followed for 15 days. N=3 for the 39 and 300 pfu groups, N=2 for the 3000 pfu group.

Figure 3: GP Mortality After PIC Infection.

Data is a compilation from 5 experiments. Strain 13 guinea pigs were inoculated with 3000 pfu adPIC or PIC 3739. They were sacrificed when moribund (>25% loss of original body weight, loss of febrile response). The post-inoculation interval at which these criteria were achieved was counted as the day of death. N=at least 12 for all points, except adPIC, day 12, in which only 5 animals were kept until day 12.

Figure 4: PIC Infection of Macrophages: Ex Vivo Focus Forming Assay.

Focus forming assays were performed as described in the text. Data is from a single experiment. Each bar represents the mean and standard deviation of separate cultures from individual animals, where n=3 for adPIC day 6 cultures, n=6 for PIC3739 day 6 cultures, and n=2 for all day 11 cultures.
SM=splenic macrophages; PM=peritoneal macrophages. * Statistically significant difference between strains, $p < .05$.

Figure 5: Serum TNF in GP adPIC Infection.

Data represents a compilation from 7 separate experiments, and is shown in tabular form in Table 3. TNF was measured by the L929 cell bioassay; laboratory units were converted to pg TNF by comparison with a concurrently assayed recombinant murine TNF standard. TNF was undetectable in normal guinea pig serum controls (not shown). Serum was collected in siliconized tubes and was not heated prior to assay.
* Significantly different from PIC 3739 strain, $p < .05$ (Mann-Whitney U-test).
"u.d."= undetectable.

Figure 6: TNF in Spleen in PIC Infection.

Spleen homogenates were prepared as 10% w:v suspensions in tissue culture media. Data is derived from a single experiment. Each bar represents the mean for three animals, except day 6, PIC 3739, which includes four animals. Results are reported as units of TNF per ml of homogenate, equivalent to units TNF per 100 mg spleen tissue.

Figure 7: TNF Production by LPS Treated PEC in PIC Infection.

500 gram male strain 13 guinea pigs were inoculated intraperitoneally with 3000 pfu of either virus strain on day 0. Peritoneal macrophages (A) or splenic macrophages (B) were isolated by plastic adherence and cultured in the presence of 10μg/ml E. coli LPS for 24 hours. Supernatants were harvested and assayed for TNF. Each bar represents the mean of macrophage cultures from three guinea pigs. Control cultures from three uninfected guinea pigs were established under identical conditions in parallel with PIC infected cultures.
* Significantly different from control, $p < .05$ (Mann-Whitney U-test).

Figure 8: Neutralization of Murine and GP TNF by Rabbit Anti-Murine TNF Antiserum.

Supernatants from mouse or guinea pig peritoneal macrophage cultures stimulated with 0.1 μg/ml or 10 μg/ml E. coli LPS respectively were mixed with indicated dilutions of rabbit anti-murine TNFα antiserum (Genzyme). Residual TNF activity was measured according to the standard L929 cytotoxicity assay. Results are reported as percentage decrease in TNF activity compared to supernatants pre-incubated with media in lieu of antiserum. Supernatants pre-incubated with normal guinea pig serum had equivalent TNF levels to those of un-pretreated supernatants.

Figure 9: Neutralization of Human and GP TNF by Anti-Human TNF Antiserum.

Dilutions of natural human TNF or TNF-containing guinea pig PM supernatants were pre-incubated for 60 minutes with indicated dilutions of polyclonal rabbit anti-human TNF antiserum or preserum (Endogen). Samples were assayed on L929 cells for residual TNF activity. Any reduction in TNF activity in samples pre-incubated with preserum was subtracted from neutralization by antiserum to yield "specific neutralization".

Figure 10: Purification of Guinea Pig TNF by HPLC.

The top, solid line represents OD_{215} and the bottom curve shows TNF bioactivity on a log scale. The large bifid peak just before the TNF peak elutes with a human serum albumin standard. The small arrow marks the protein peak showing maximum TNF activity.

Figure 11: Prostaglandin Production by PEC During adPIC Infection.

Data from 2 separate experiments are shown in Figures 11A and B. Experiments were performed exactly as described in Figure 7. Supernatants were assayed for PGE_2 by ELISA as described.

Figure 12: PIC Titers in Blood and Spleen.

500 gram male strain 13 guinea pigs were inoculated intraperitoneally with 3000 plaque forming units of either adPIC of PIC3739. On the indicated post-inoculation day, 3 animals from each group (4 from day 6, PIC3739) were killed. Viral quantitations were performed on whole anticoagulated blood **(A)** or 1:10 (w:v) homogenates of spleen in tissue culture media **(B)**.

Figure 13: Peritoneal Macrophage Numbers During PIC Infection.

Data is a compilation from two separate experiments in which the two PIC strains were directly compared. Peritoneal exudate cells were obtained by a standard method and the proportion of macrophages was determined by examination of Diff-Quick stained cytospins. Recovery was calculated as a percent of the mean number of PM obtained from 5 sham-injected guinea pigs.

Figure 14: PIC Replication in Peritoneal Macrophages After In Vitro Infection.

Mineral oil elicited peritoneal macrophages from normal strain 13 guinea pigs were infected in vitro with adPIC or PIC3739 at multiplicities of infection of 1.0, 0.1, and 0.01 and cultured in the presence **(B)** or absence **(A)** of E. coli LPS as described in the text. Cell-free supernatants were harvested at indicated time points and assayed for infectious virus and TNF. TNF production relative to control ("sham" infected) cultures is indicated by symbols above the relevant curve.

* TNF overproduction compared to control; # TNF underproduction compared to control.

Figure 15: LPS Induced TNF Production by PEC After In Vitro PIC Infection.

Same experiment as Figure 15. Mineral oil elicited macrophages were infected at given moi with adPIC or PIC3739, and cultured for the duration of the experiment in the presence of E. coli LPS (10 μg/ml). Supernatants were harvested at indicated time points and cells removed by centrifugation before assaying for TNF. Solid bars represent adPIC infected macrophages, cross-hatched bars represent PIC3739 infected macrophages.
+ —— + parallel control (sham-infected) culture.

Figure 16: PIC Killing by LPS Treated PEC.

The same experiment is depicted as in Figures 15 and 16. The log of the difference in viral yields between LPS treated and non-LPS treated macrophage cultures infected with PIC at a given moi is plotted. Solid bars represent adPIC infected macrophages, cross-hatched bars represent PIC3739 infected macrophages.

Figure 17: Lymphocyte Proliferation to PIC Antigens in PIC3739 Immunized Guinea Pigs.

500 gram male strain 13 guinea pigs were immunized with the indicated dose of PIC3739, and either challenged with 10^4 pfu adPIC on day 25 ("challenge"), or not treated ("no challenge"). Animals were killed on day 59, and spleen cells were placed in culture with dilutions of UV-killed adPIC which was concentrated from tissue culture fluid by polyethylene glycol precipitation. Data shown here reflect lymphoproliferative responses at 5 days **(A)** and 7 days **(B)** to the optimal antigen concentration, approximately 0.02 pfu equivalent per spleen cell. Stimulation index equals mean ^{3}H-thymidine uptake (cpm) of sample divided by background ^{3}H-thymidine uptake by unstimulated spleen cells cultured in media alone. Peak concanavalin A induced proliferation and background counts were comparable for all cultures. Each bar represents mean of triplicate cultures from a single guinea pig. Controls are described in Figure 18.

Figure 18: Lymphocyte Proliferation Experiment #2.

The experiment was conducted as described in Figure 1, except that animals were killed on day 82 (immunized day 0, challenged day 25). 5 day **(A)** and 7 day **(B)** lymphocyte proliferation assays were performed. Controls included two normal, naive guinea pigs. "Mock" antigen (PEG precipitated conditioned Vero cell supernatant) elicited no proliferative response (not shown). Each bar represents mean of triplicate cultures from a single guinea pig, except the last bar, which is the mean of triplicate cultures from two normal control animals.

Figure 19: Western Immunoblot of PIC Antigens.

Pichinde virus proteins in PEG concentrated tissue culture supernatants were separated on an 8% SDS polyacrylamide gel and developed with polyclonal antiserum to PIC3739 raised in guinea pigs. Lane 3 represents control, PEG precipitated supernatant from mock-infected Vero cells.

Figure 20: Responses to Concanavalin A of Splenocytes Isolated from Animals on Different Days After Pichinde Virus Inoculation.

Strain 13 guinea pigs were inoculated intraperitoneally with 2 x 10^4 PFU of Pichinde virus, or RPMI 1640. Splenocytes were then isolated from these animals **(A)** 3, **(B)** 7, and **(C)** 11 days after inoculation and were cultured with concanavalin A for 72 hours. Proliferative responses were determined by ^{3}H-labeled thymidine incorporation. Each bar represents mean counts per minute of triplicate cultures from a single guinea pig. The data are expressed as mean counts per minute ± 1 SD.

Figure 21: Responses to T-Cell Mitogens of Peripheral Blood Mononuclear Cells Isolated from Animals 7 Days After Viral Infection.

Strain 13 guinea pigs were inoculated intraperitoneally with $2x10^4$ PFU of Pichinde virus, or RPMI 1640. Blood was collected from animals by cardiac puncture, treated with heparin, and then diluted 1:2 with Hanks buffered saline solution. Peripheral blood mononuclear cells were isolated by centrifugation of the diluted blood over Ficoll-Hypaque. Isolated cells were then cultured with **(A)** concanavalin A or **(B)** phytohemagglutinin for 72 hours. Proliferative responses were determined by ^{3}H-labeled thymidine incorporation. The data are expressed as mean counts per minute of triplicate cultures ± 1 SD.

Figure 22: Responses to Concanavalin A of Peripheral Blood Mononuclear Cells Isolated from Animals 11 Days After Viral Infection.

Strain 13 guinea pigs were inoculated intraperitoneally with $2x10^4$ PFU of adPIC, or RPMI 1640. Blood was collected from animals by cardiac puncture, treated with heparin, and then diluted 1:2 with Hanks buffered saline solution. Peripheral blood mononuclear cells were isolated by centrifugation of the diluted blood over Ficoll-Hypaque. Isolated cells were then cultured with concanavalin A for 72 hours. Proliferative responses were determined by ^{3}H-labeled thymidine incorporation. The data are expressed as mean counts per minute ± 1 SD of triplicate cultures.

Figure 23: Effect of Incubation Time on Responses of Splenocytes from Infected and Control Animals to Concanavalin A.

Strain 13 guinea pigs were inoculated with $3x10^3$ PFU of adPIC or RPMI 1640. Splenocytes were then isolated from these animals 11 days after inoculation and were cultured with concanavalin A for **(A)** 120, **(B)** 96, or **(C)** 72 hours. Proliferative responses were determined by ^{3}H-labeled thymidine incorporation. The data are expressed as mean counts per minute ± 1 SD of triplicate cultures.

TABLE 1: adPIC Infection in Outbred Guinea Pigs.

Male Hartley guinea pigs were infected with 3000 pfu adPIC by the intraperitoneal route as described in the text.
* culture positive for E. coli
♦ culture positive for Proteus mirabilis
◙ culture positive for Pseudomonas aeruginosa

TABLE 2: Dose Response of Strain 13 GP to PIC 3739.

Male strain 13 guinea pigs (500 grams) were inoculated with the indicated amount of PIC 3739 subcutaneously on day 0. All animals recovered after infection.

TABLE 3: Serum TNF in GP Infected with PIC Strains.

Data represents compilation from 7 separate experiments. Strain 13 guinea pigs (400-600 grams) were used except as noted below. All guinea pigs were infected with 3000 pfu of PIC by the intraperitoneal route. TNF levels are expressed as mean pg/ml ± standard deviation. Data points listed as "0" are below the limits of detection of the L929 bioassay (ranging from 0.6 pg/ml to 200 pg/ml). In the column labelled #pos/n, the numerator represents the number of animals with detectable TNF in their serum, and the denominator denotes the total number of animals in the group.

§ This group includes 2 outbred Hartley guinea pigs (one with detectable TNF).
† This group includes 5 outbred guinea pigs (one positive).
‡ This group consists of a single outbred Hartley guinea pig.
ł This group consists of 8 strain 2 guinea pigs.
* Statistically significant difference between two PIC strains, $p < .05$, Mann-Whitney U-Test.
"n.d." = not done.

TABLE 4: Relative Amounts of TNF mRNA in Guinea Pigs Organs During adPIC Infection.

Determination of relative levels of TNF mRNA was by slot blot as described in Materials and Methods. Organ RNA samples were from guinea pigs 6 days after inoculation with 3000 pfu adPIC, or 2.3 hours after inoculation with a lethal dose of E. coli LPS (20 mg/kg). RNA from untreated guinea pig organs served as the normal baseline for comparison. Guinea pig peritoneal macrophages were stimulated for 2 hours with E. coli LPS (10μg/ml) before isolating RNA; unstimulated PM were used as control for baseline. All densitometry data was normalized for ß-actin mRNA content.

TABLE 5: Partial Purification of Guinea Pig TNF.

TABLE 6: Histologic Features Associated with Lethality in PIC Infection.

Strain 13 guinea pigs were inoculated with 3000 pfu adPIC or PIC3739, and sacrificed on day 11, 12, or 15. Guinea pigs were classified as "moribund" (group I) if they had lost 25% or more of initial body weight and had lost a febrile response. This group included all of the adPIC infected animals and 2 PIC3739 animals at day 15. Group II included "recovering" animals that failed to meet the above criteria, and included the remainder of the PIC3739 group. Hematoxylin and eosin stained sections were examined from the tissues indicated. The denominator of the fraction in each column represents the number of animals from which a tissue was examined, and the numerator represents the number of animals displaying the indicated lesion. Lesions were catalogued and graded as follows:

SPLEEN: **MZN**: marginal zone necrosis/fibrinoid change; 1-3+
RPN: red pulp necrosis; 1-3+

LYMPH NODE (mesenteric)(LN): **H**: Sinus histiocytosis
N: Necrosis

LIVER: **F**: fatty change; 1-2+ mild, mid-zonal; 3+ severe, panlobular
PI: portal inflammation; 1+ mild, portal tract not expanded; 2+ moderate, portal tract expanded; 3+ severe, expansion of portal tract and piecemeal necrosis of limiting plate
LI/HN: lobular inflammation/hepatocyte necrosis; 1+ > 1 focus per five 10 x fields; 2+ 1-5 foci per 10 x field; 3+ > 5 foci per 10 x field.

LUNG: **IP**: interstitial pneumonitis, 1-3+

GUT (small intestine): **FM**: foamy macrophages in the lamina propria; 1+ lamina propria not expanded; 2-3+ lamina propria expanded, villi broad.
CY: cytoclastic debris in lamina propria macrophages, LP necrosis; 1-3+
VF: villous flattening; 1+, slight broadening; 2+ intermediate; 3+ flat villi.

TABLE 7: Protection of GP Against Lethal adPIC Infection by Immunization with PIC3739.

TABLE 8: PIC3739 Immunization: IFA Serology.

TABLE 9: Effect of Adherent Cells From Control and Infected GP on Lymphocytes Responses to Con A.

FIGURE 1

GUINEA PIG BODY WEIGHTS IN adPIC INFECTION

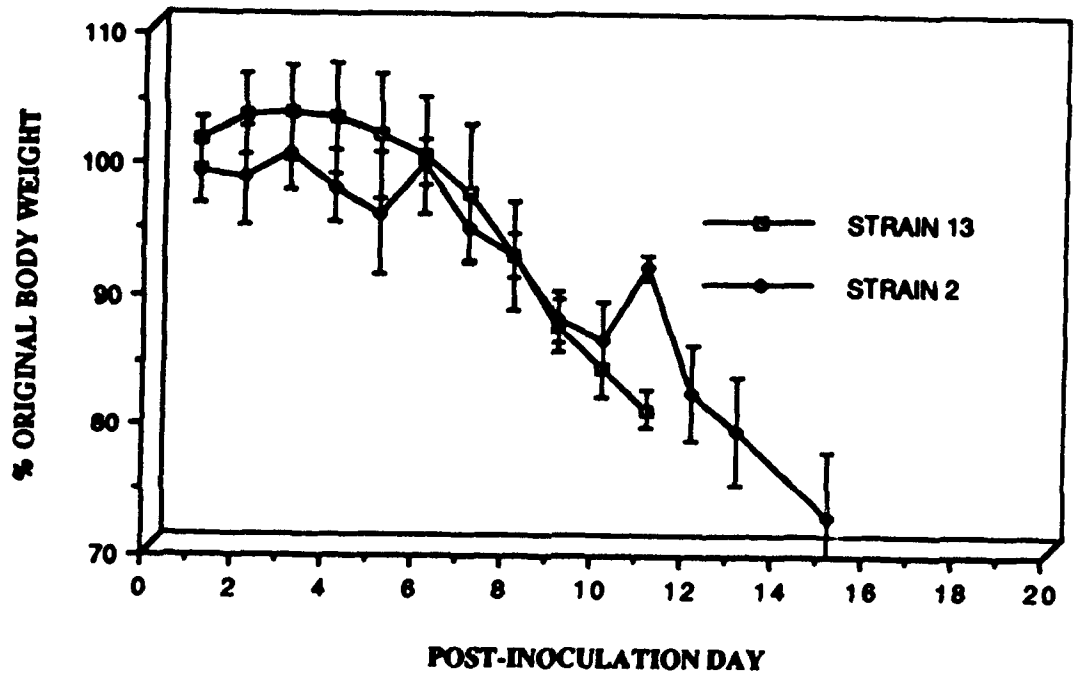

FIGURE 2

WEIGHT LOSS AND adPIC DOSE IN STRAIN 2 GP

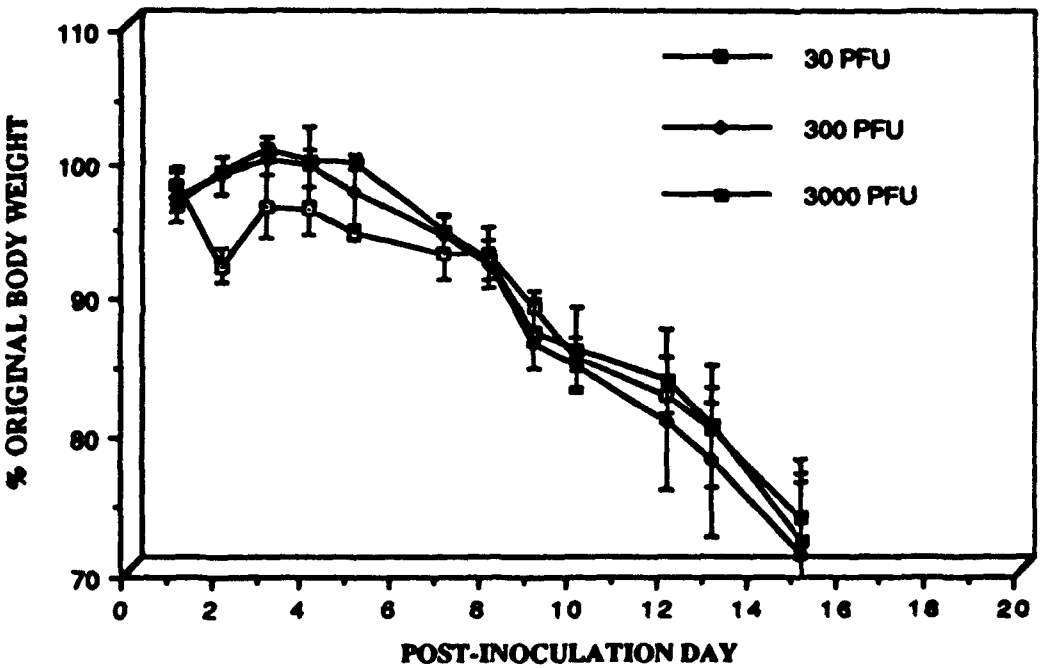

FIGURE 3

GP MORTALITY IN PIC INFECTION

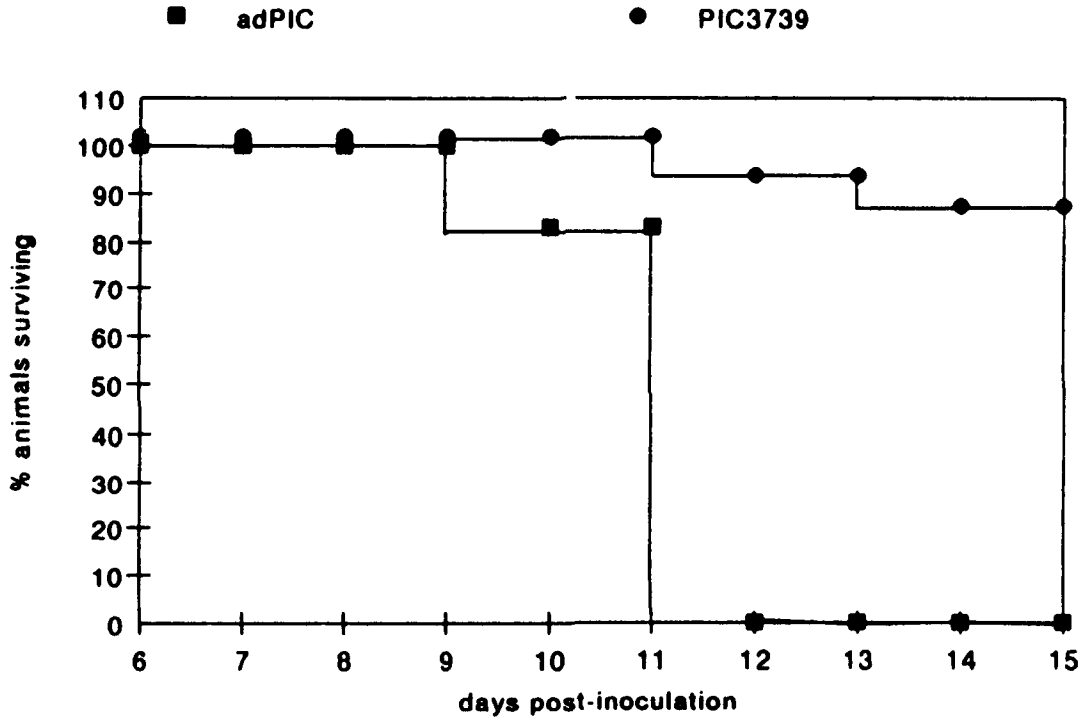

FIGURE 4

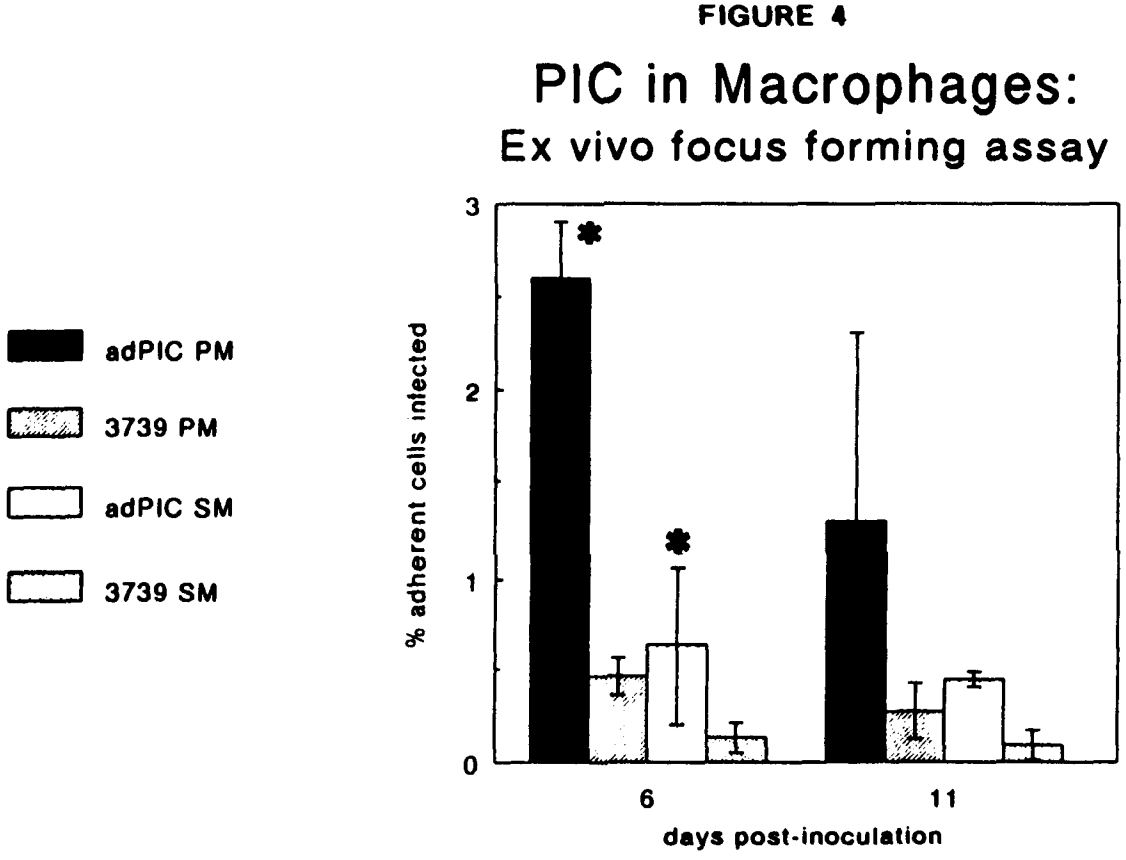
PIC in Macrophages:
Ex vivo focus forming assay
adPIC PM
3739 PM
adPIC SM
3739 SM
% adherent cells infected
0
1
2
3
6
11
days post-inoculation

FIGURE 5

SERUM TNF IN GP adPIC INFECTION

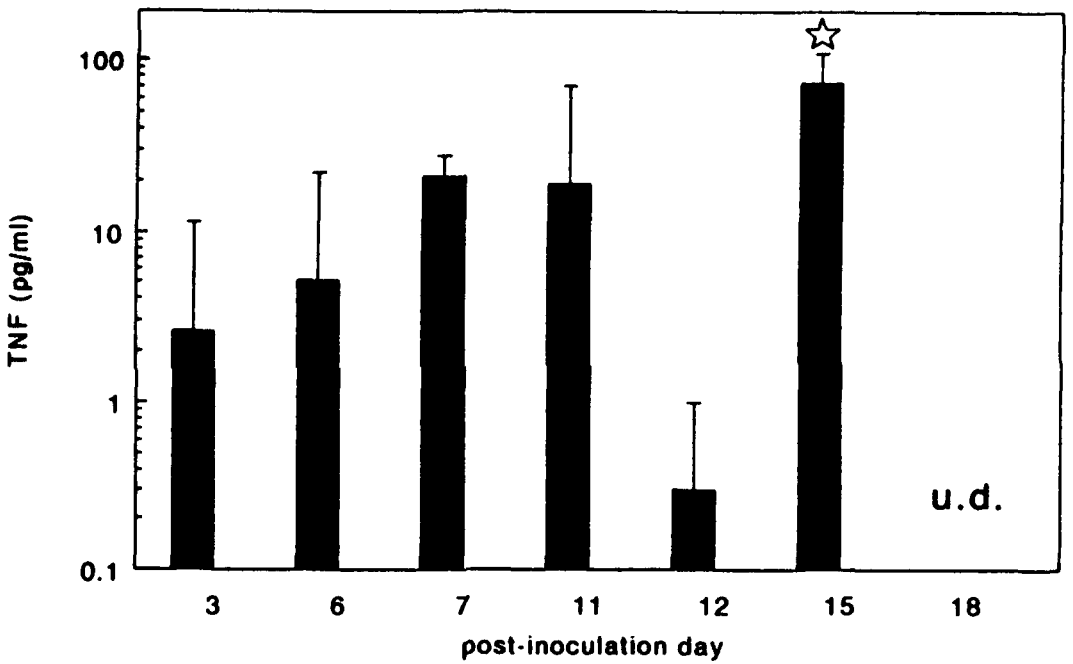

FIGURE 6

TNF IN SPLEEN IN PIC INFECTION

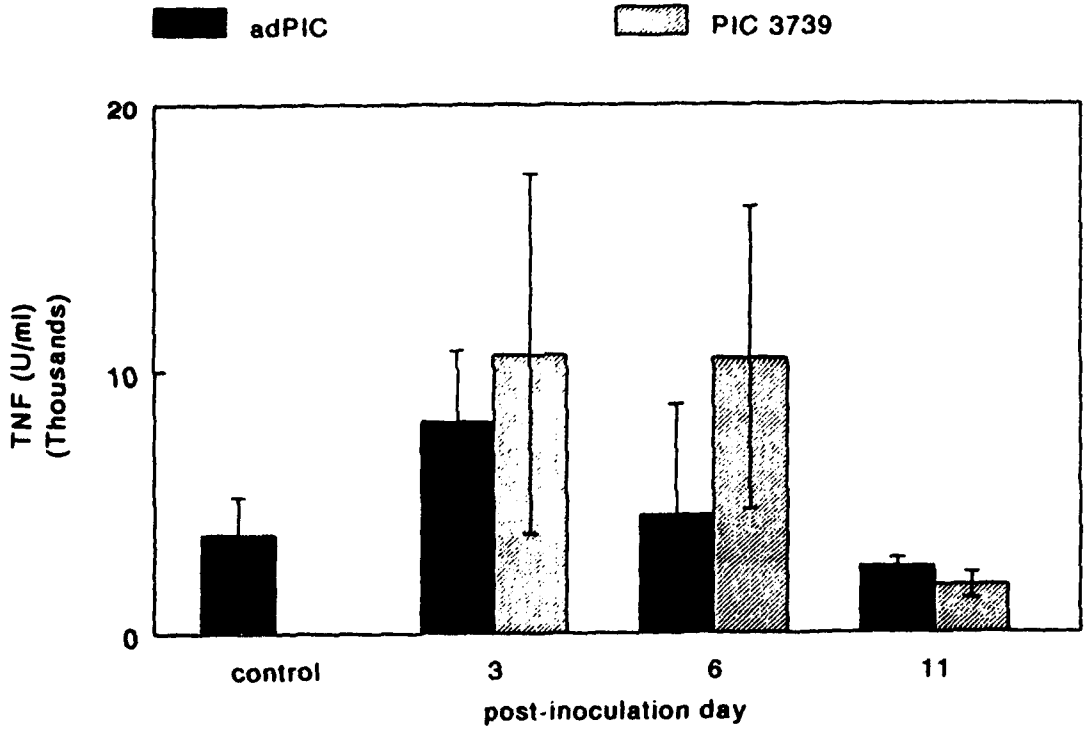

FIGURE 7A

TNF PRODUCTION BY LPS TREATED PM IN PIC INFECTION

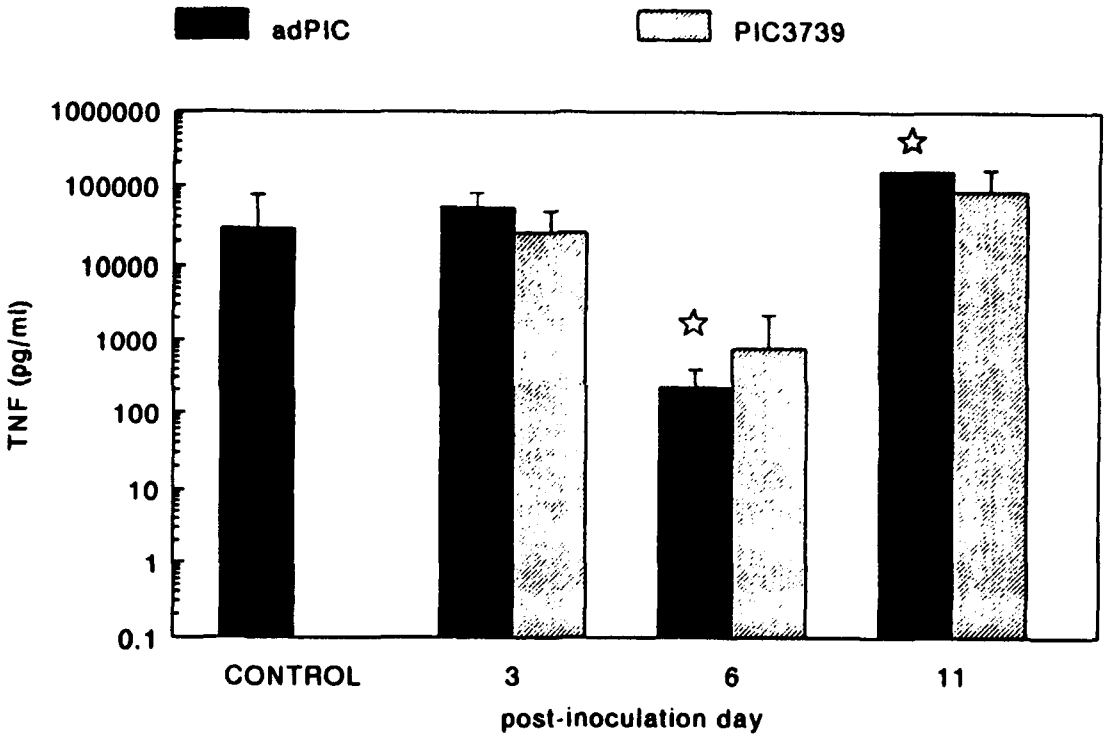

FIGURE 7B

TNF PRODUCTION BY LPS TREATED SM IN PIC INFECTION

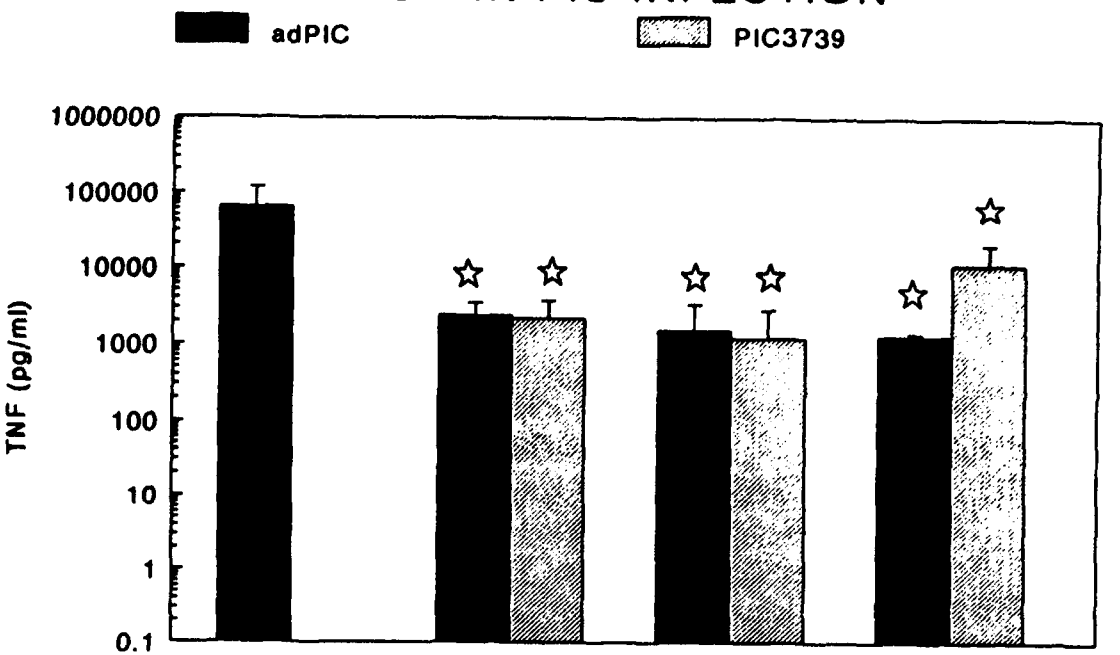

FIGURE 8

Neutralization of Murine and GP TNF

by anti-murine TNF antiserum

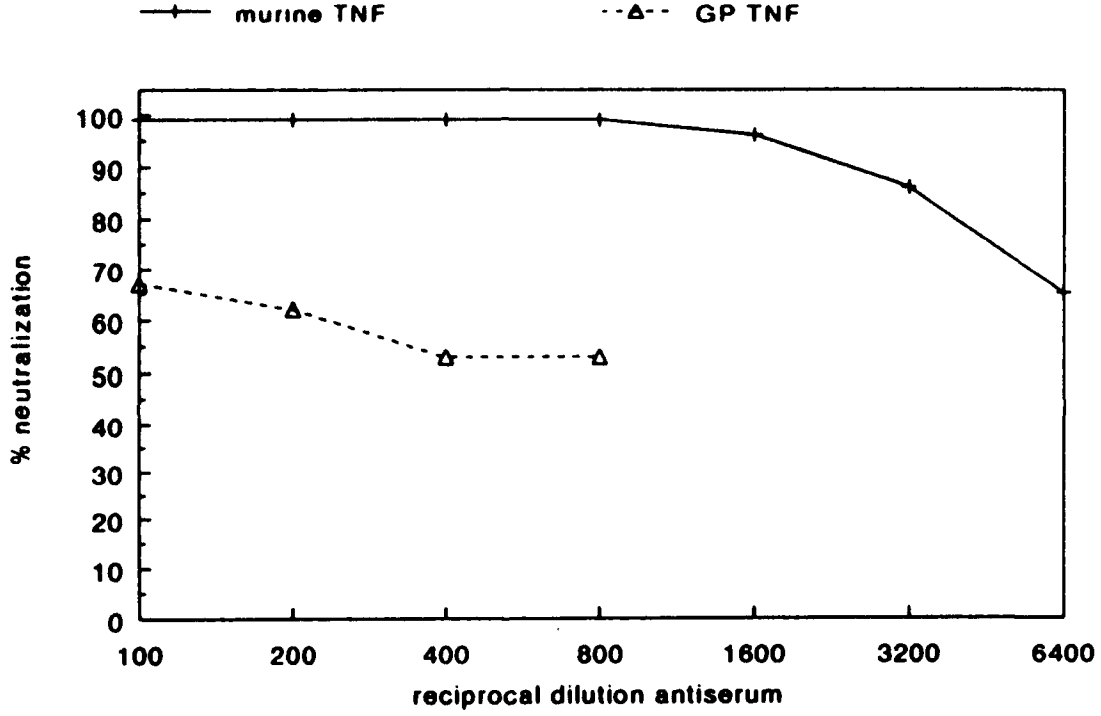

FIGURE 9

Neutralization of Hu and GP TNF

by anti-Hu TNF antiserum

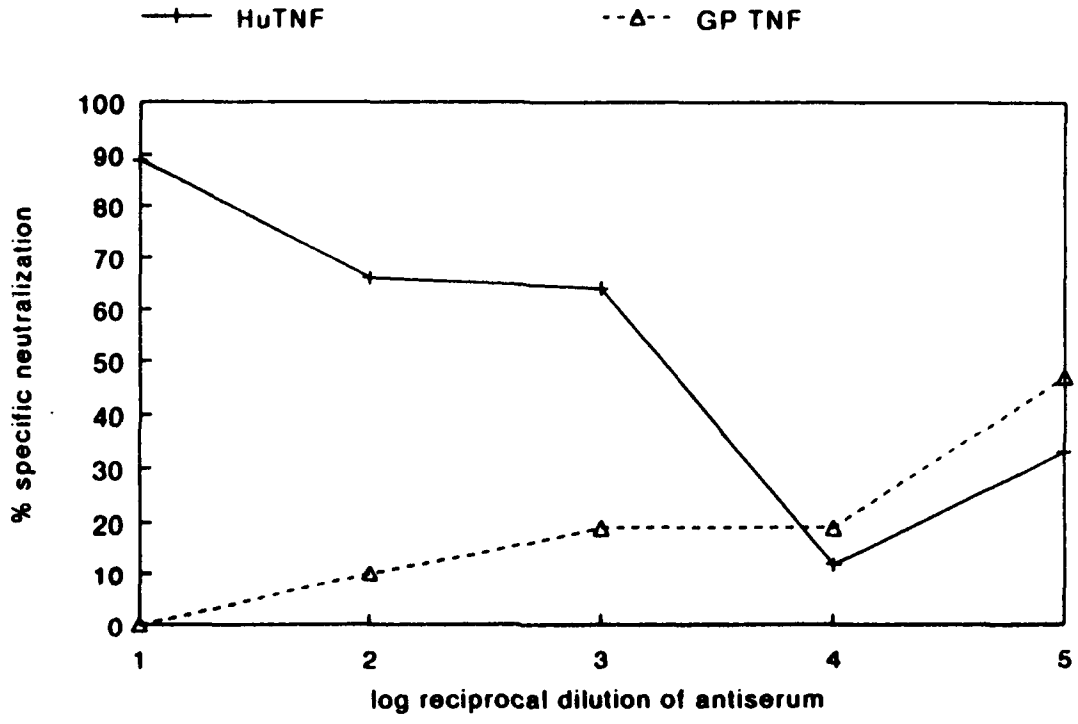

FIGURE 10

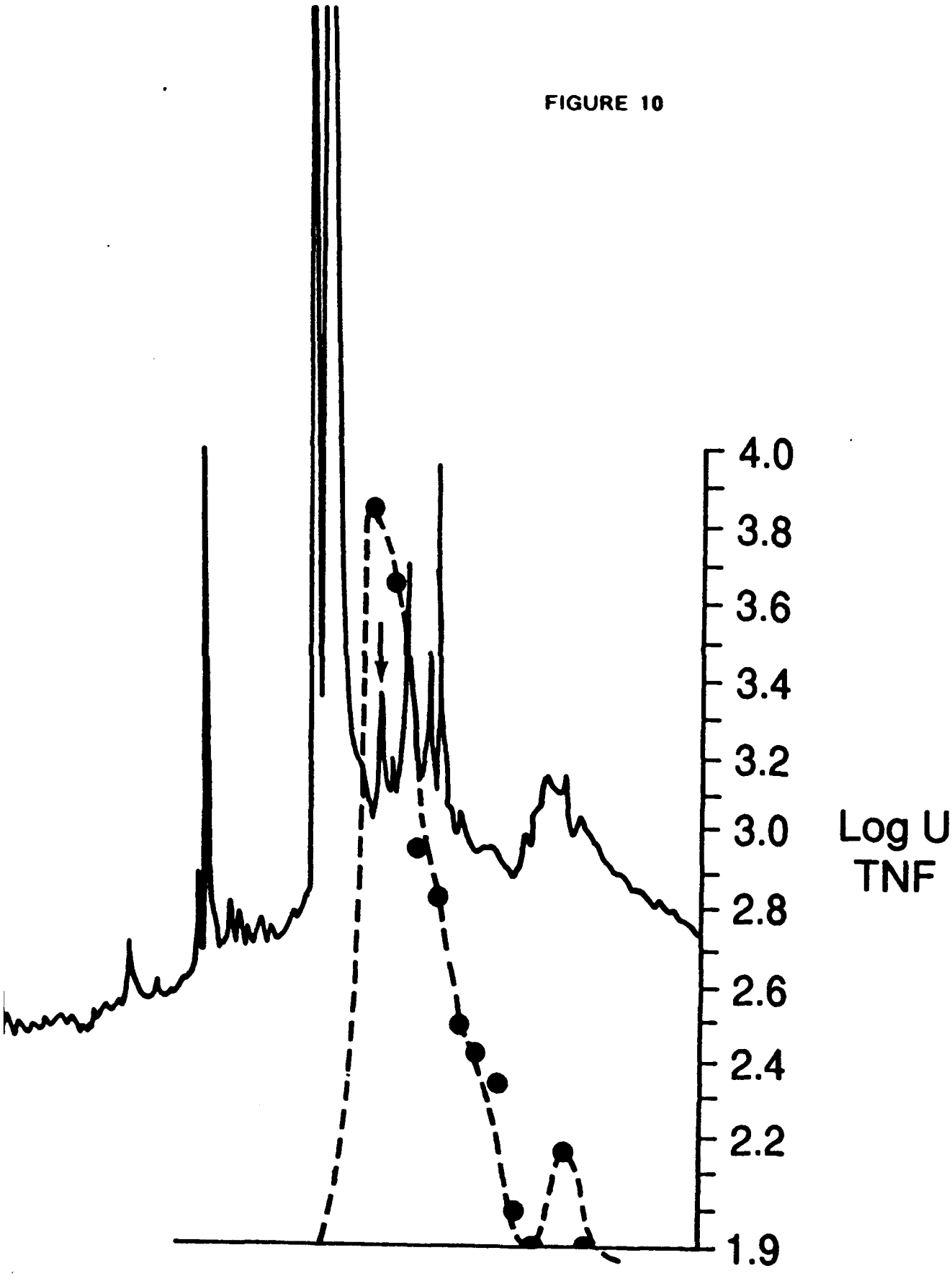

FIGURE 11A

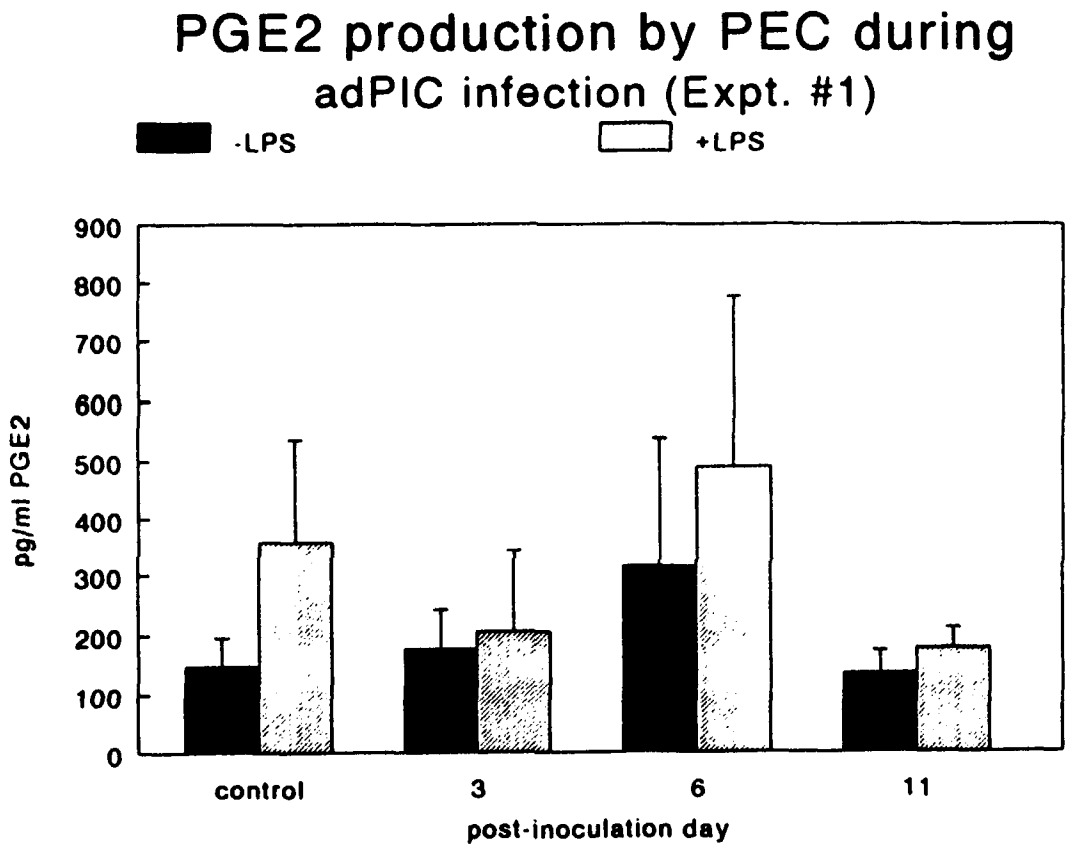
PGE2 production by PEC during
adPIC infection (Expt. #1)
-LPS
+LPS
900
800
700
600
500
400
300
200
100
0
pg/ml PGE2
control
3
6
11
post-inoculation day

FIGURE 11B

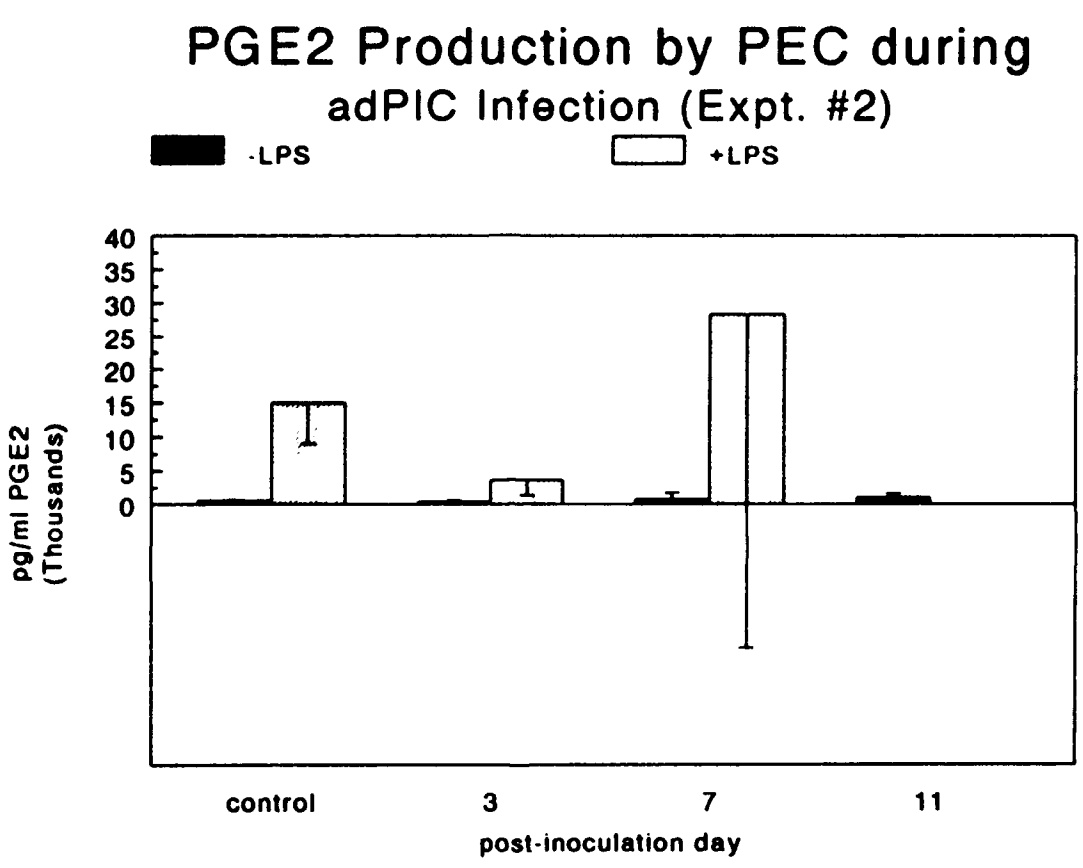
PGE2 Production by PEC during
adPIC Infection (Expt. #2)
-LPS
+LPS
40
35
30
25
20
15
10
5
0
pg/ml PGE2
(Thousands)
control
3
7
11
post-inoculation day

FIGURE 12A

PIC TITERS IN BLOOD

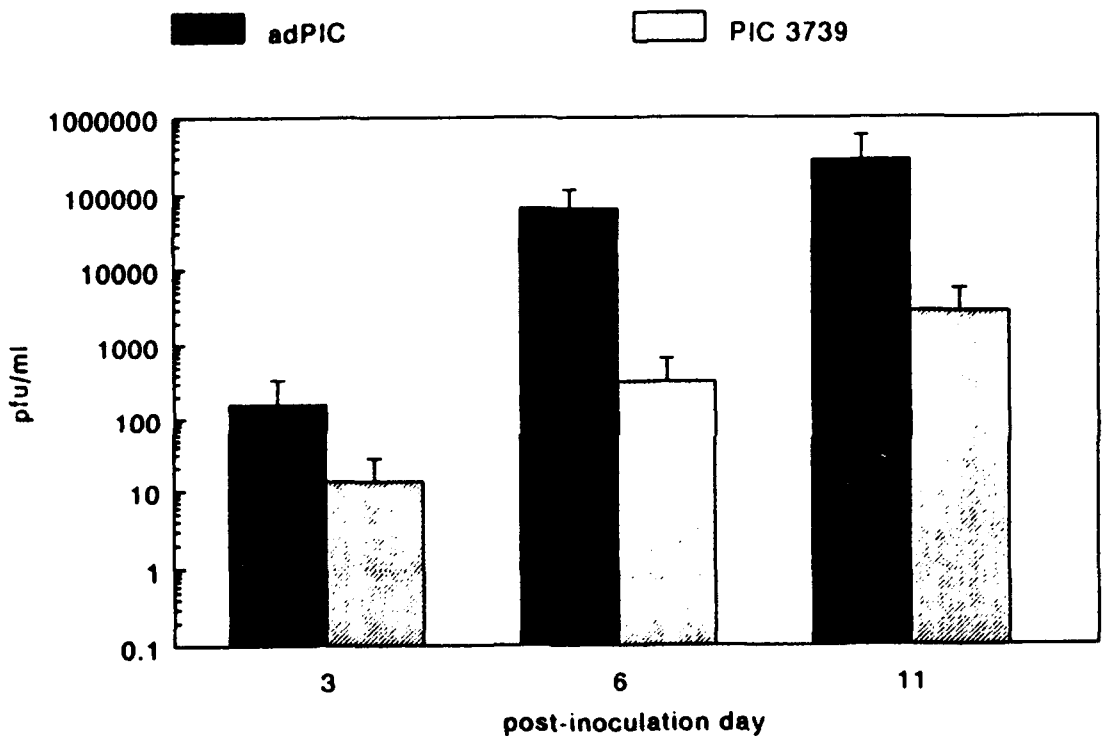

FIGURE 12B

PIC TITERS IN SPLEEN

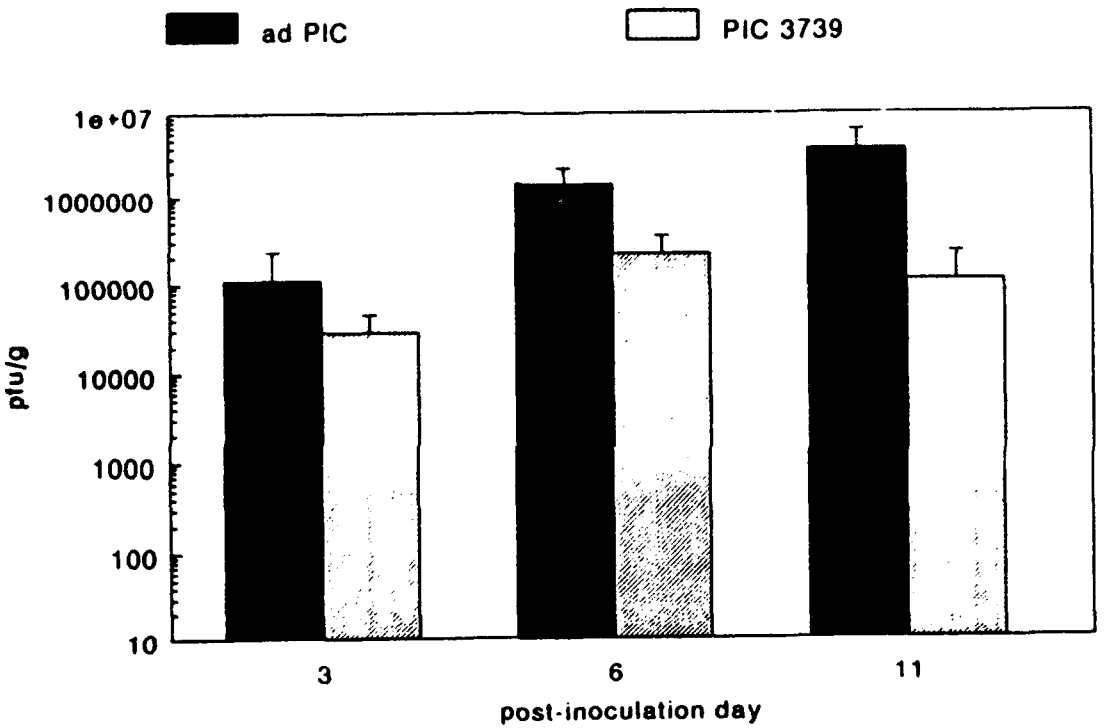

FIGURE 13

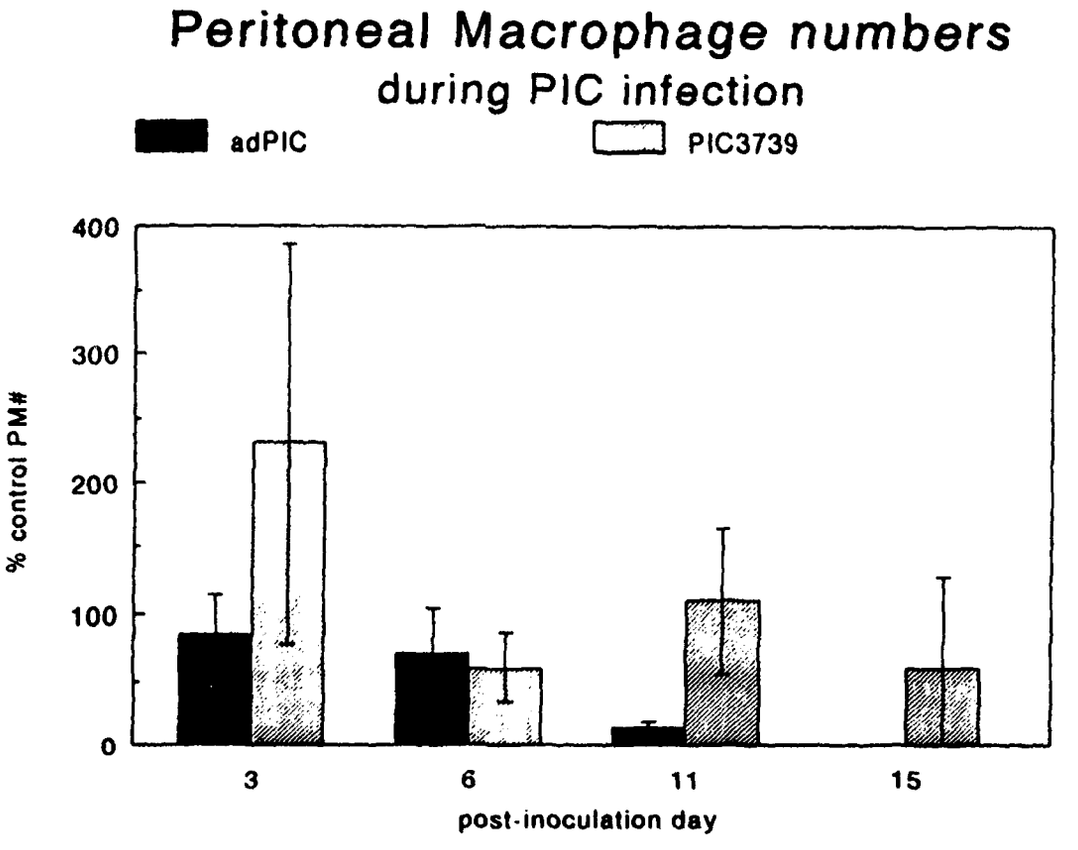
Peritoneal Macrophage numbers
during PIC infection
adPIC
PIC3739
400
300
200
100
0
% control PM#
3
6
11
15
post-inoculation day

FIGURE 14A

PIC REPLICATION IN PM

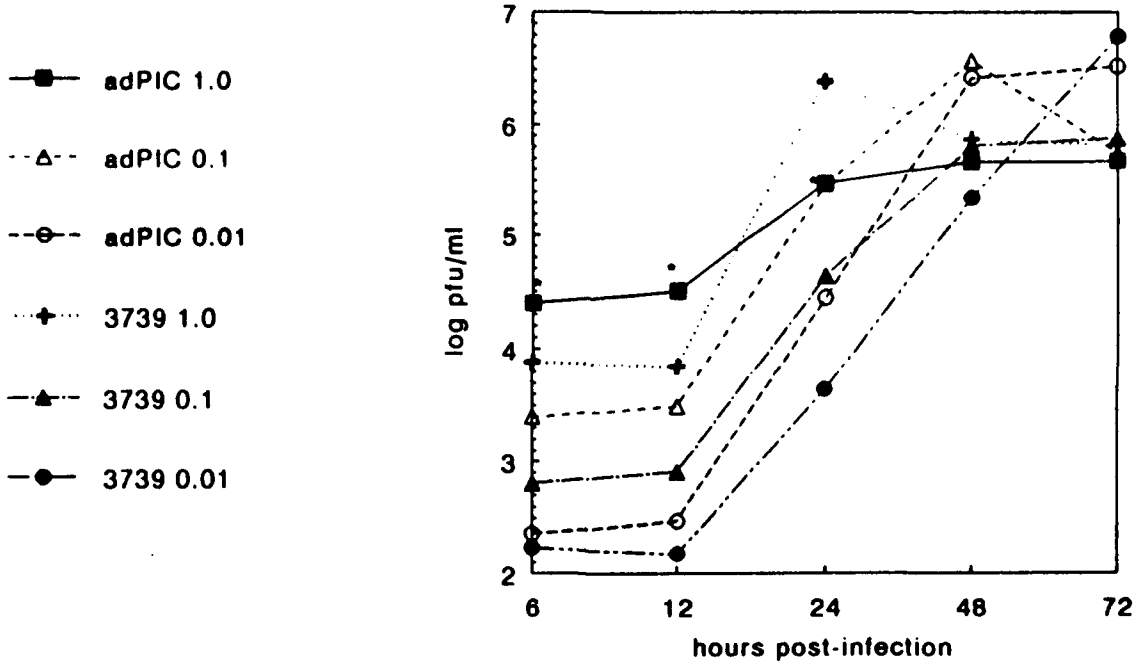

FIGURE 14B

PIC REPLICATION IN PM
after LPS

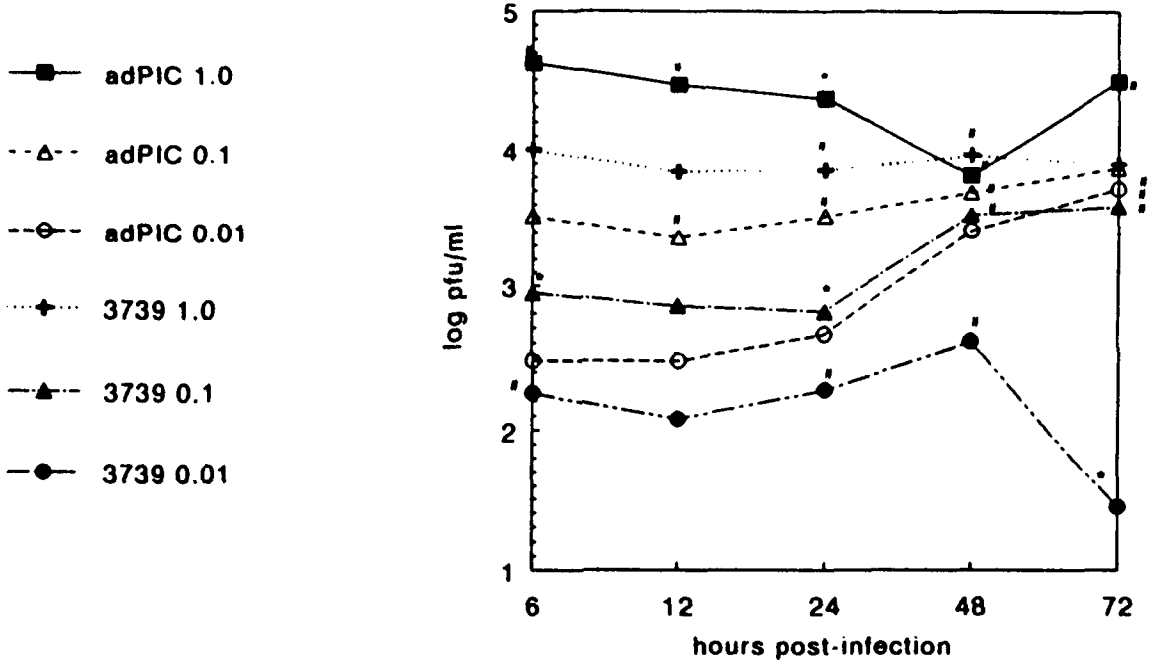

FIGURE 15

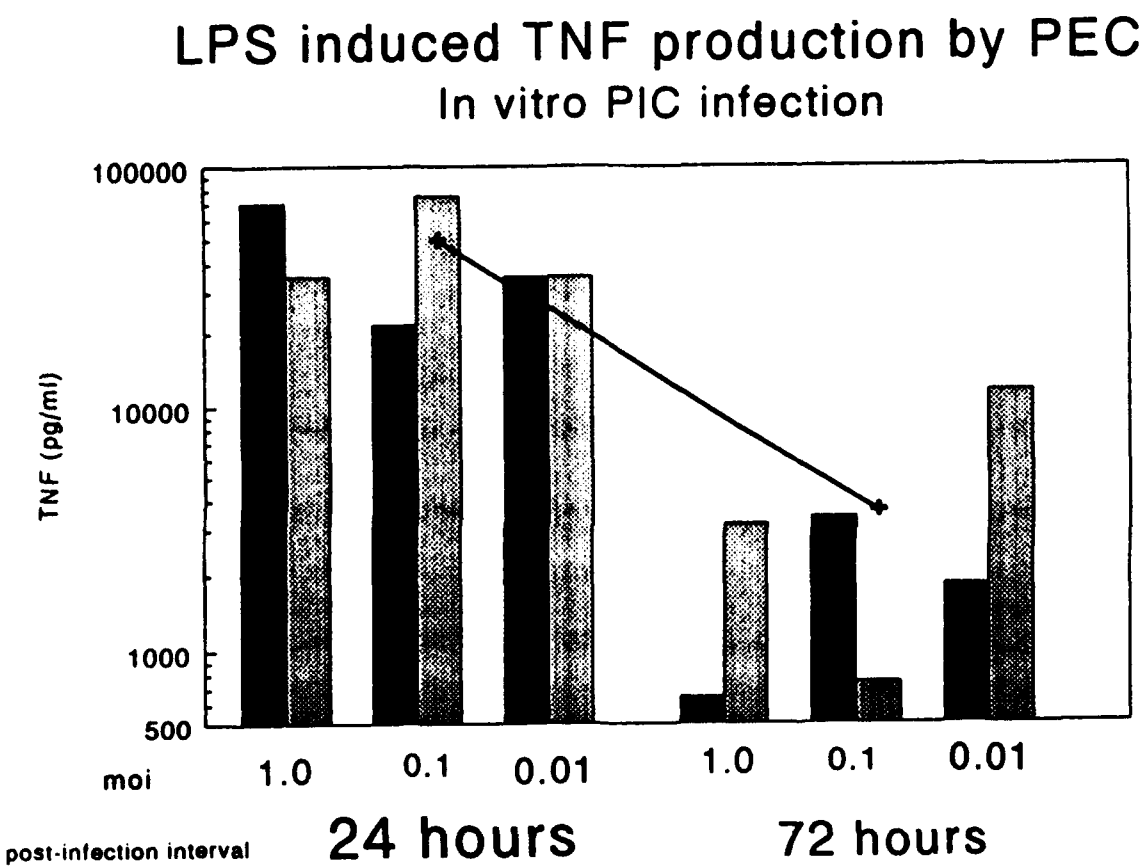
LPS induced TNF production by PEC
In vitro PIC infection
100000
10000
1000
500
TNF (pg/ml)
moi
1.0
0.1
0.01
1.0
0.1
0.01
post-infection interval
24 hours
72 hours

FIGURE 16

PIC killing by LPS treated PEC

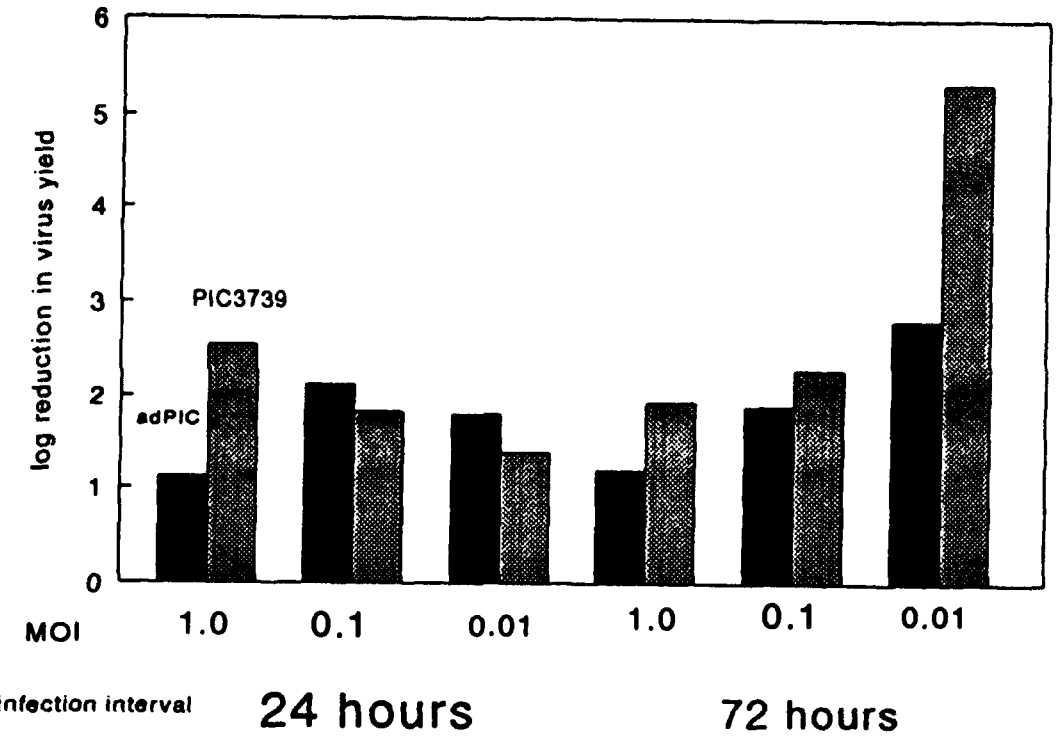

FIGURE 17A

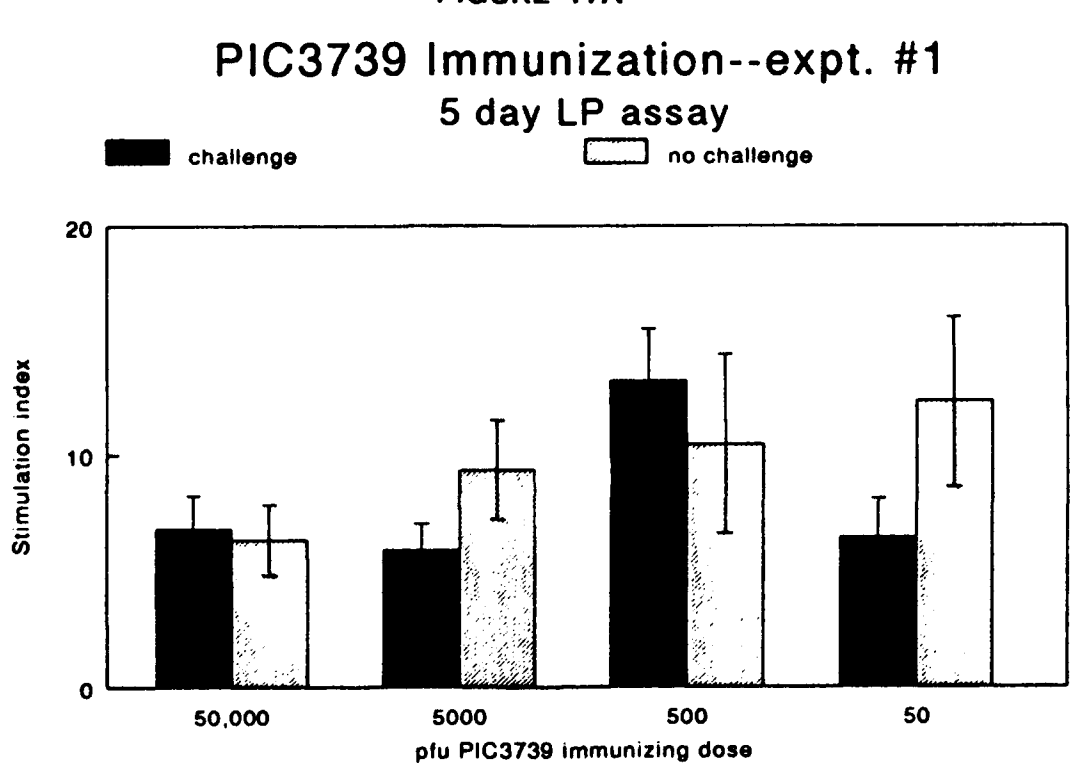
PIC3739 Immunization--expt. #1
5 day LP assay
challenge
no challenge
20
10
0
Stimulation index
50,000
5000
500
50
pfu PIC3739 immunizing dose

FIGURE 17B

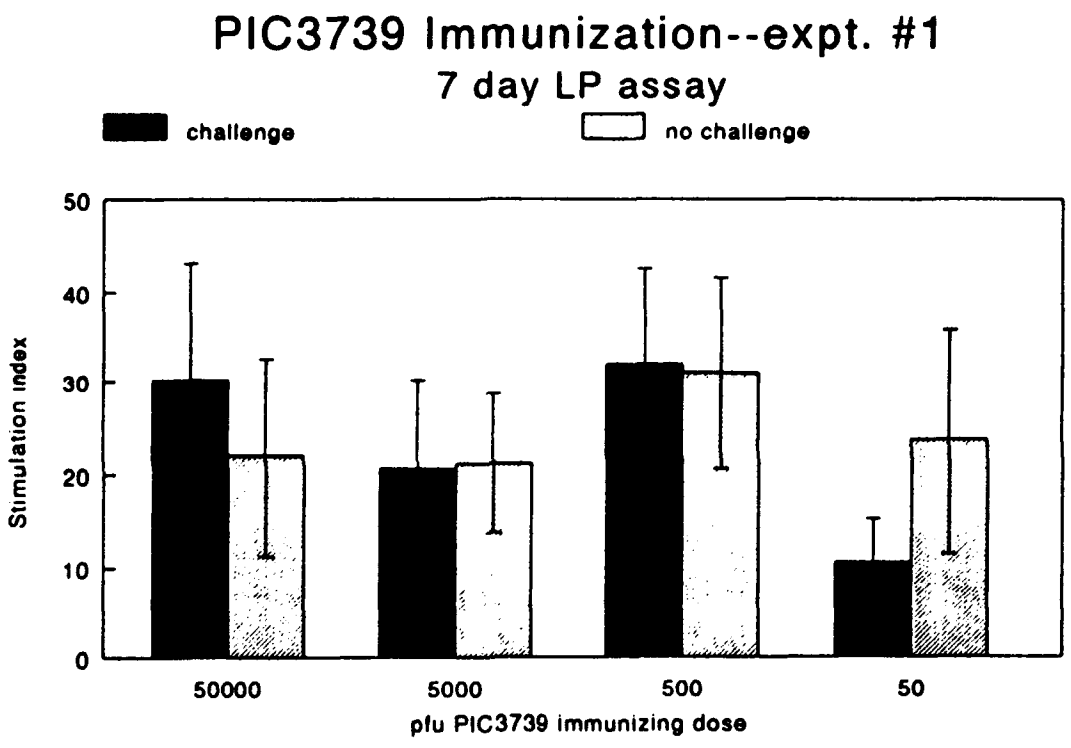
PIC3739 Immunization--expt. #1
7 day LP assay
challenge
no challenge
Stimulation index
50
40
30
20
10
0
50000
5000
500
50
pfu PIC3739 immunizing dose

FIGURE 18A

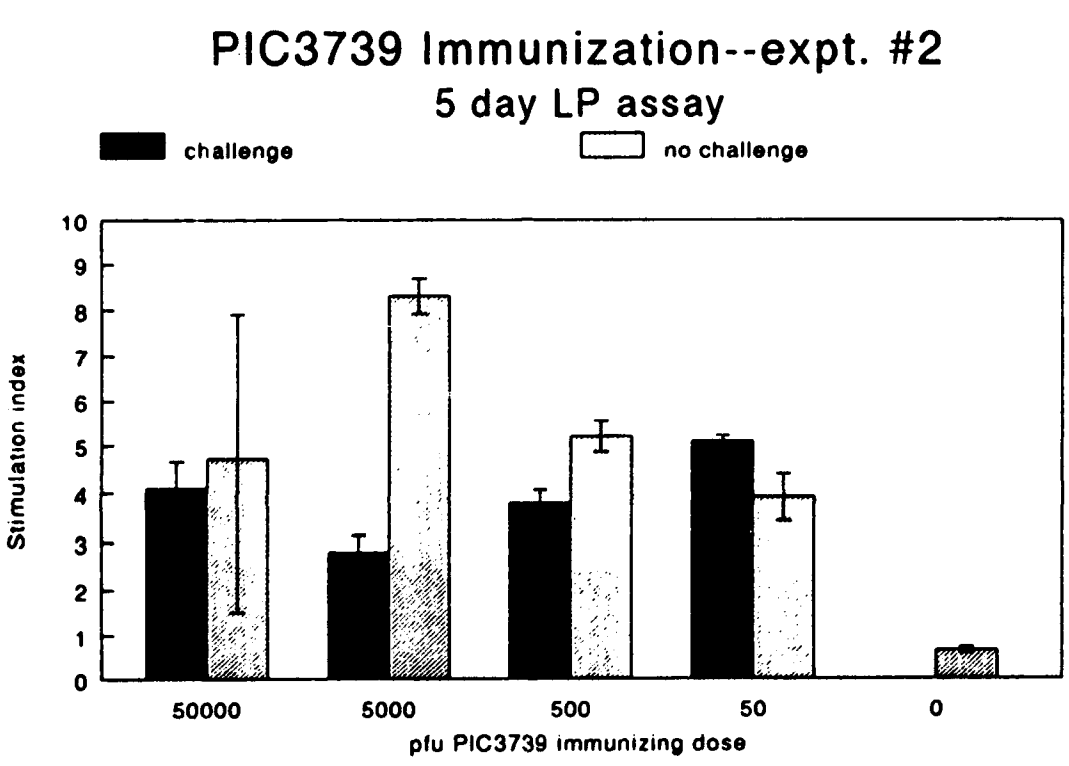
PIC3739 Immunization--expt. #2
5 day LP assay
challenge
no challenge
Stimulation index
10
9
8
7
6
5
4
3
2
1
0
50000
5000
500
50
0
pfu PIC3739 immunizing dose

FIGURE 18B

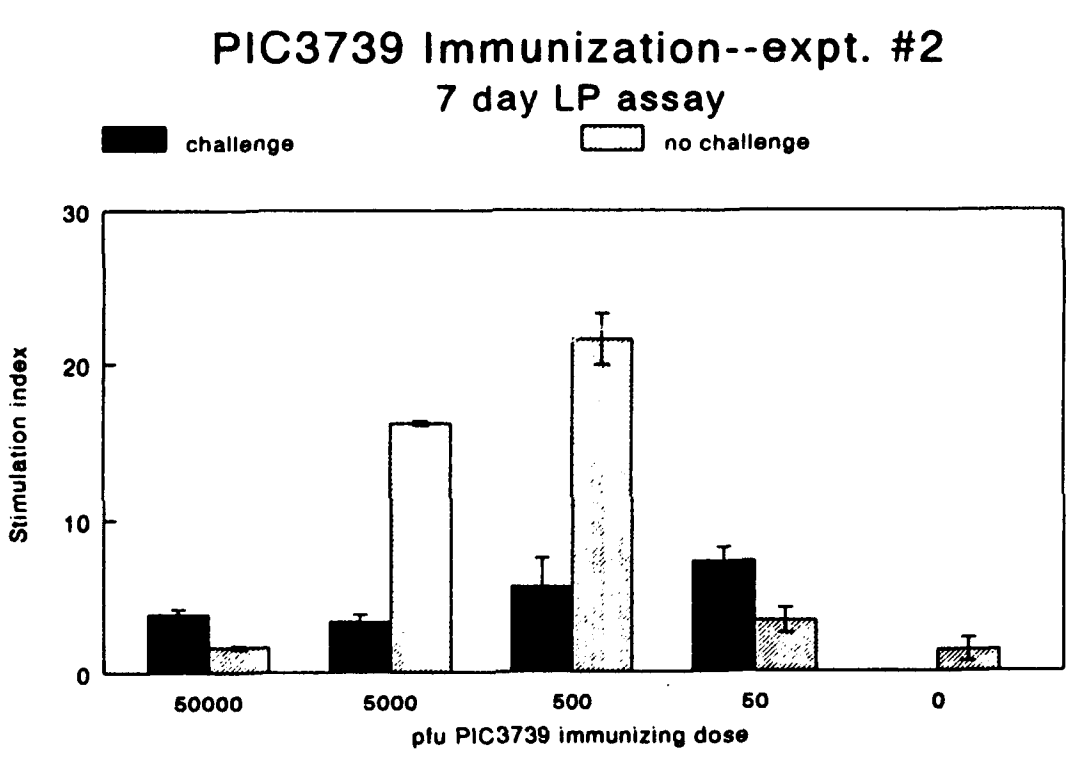
PIC3739 Immunization--expt. #2
7 day LP assay
challenge
no challenge
Stimulation index
30
20
10
0
50000
5000
500
50
0
pfu PIC3739 immunizing dose

FIGURE 19

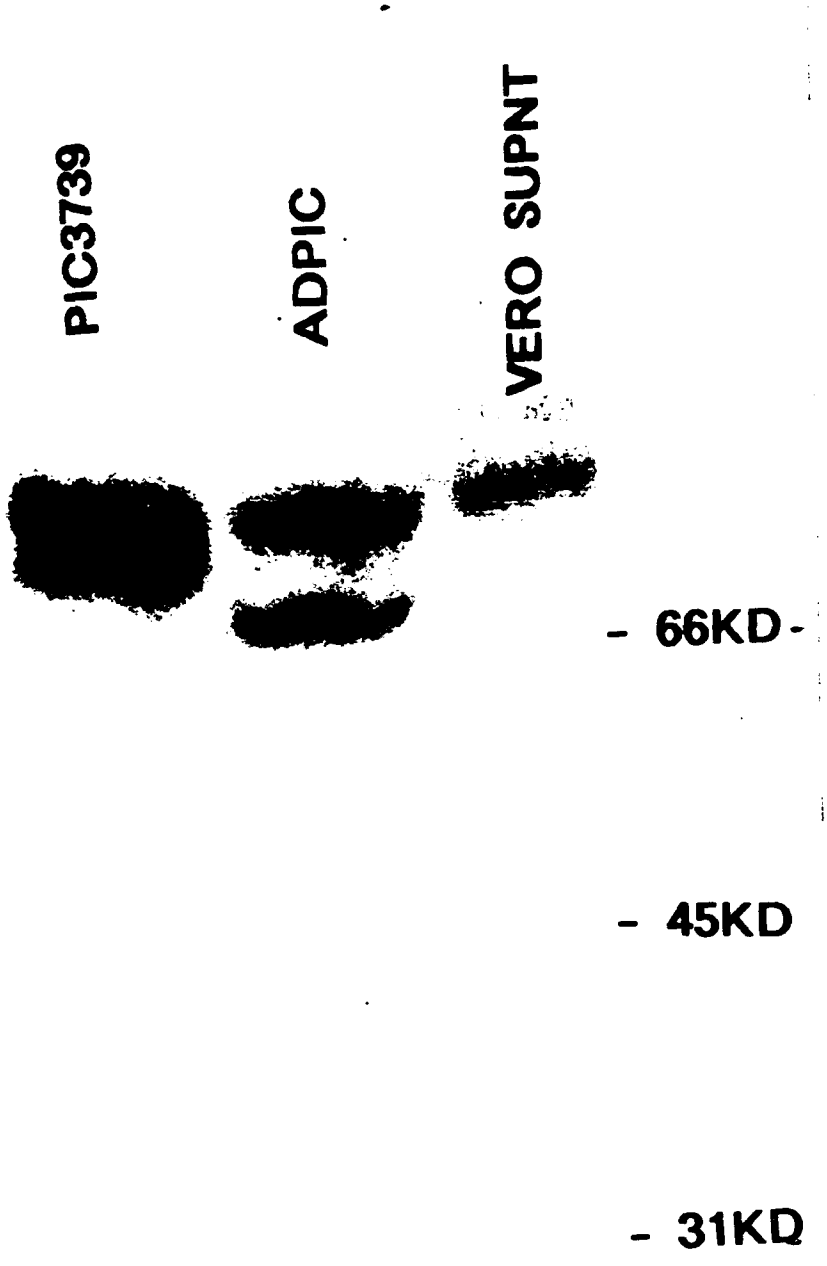

FIGURE 20

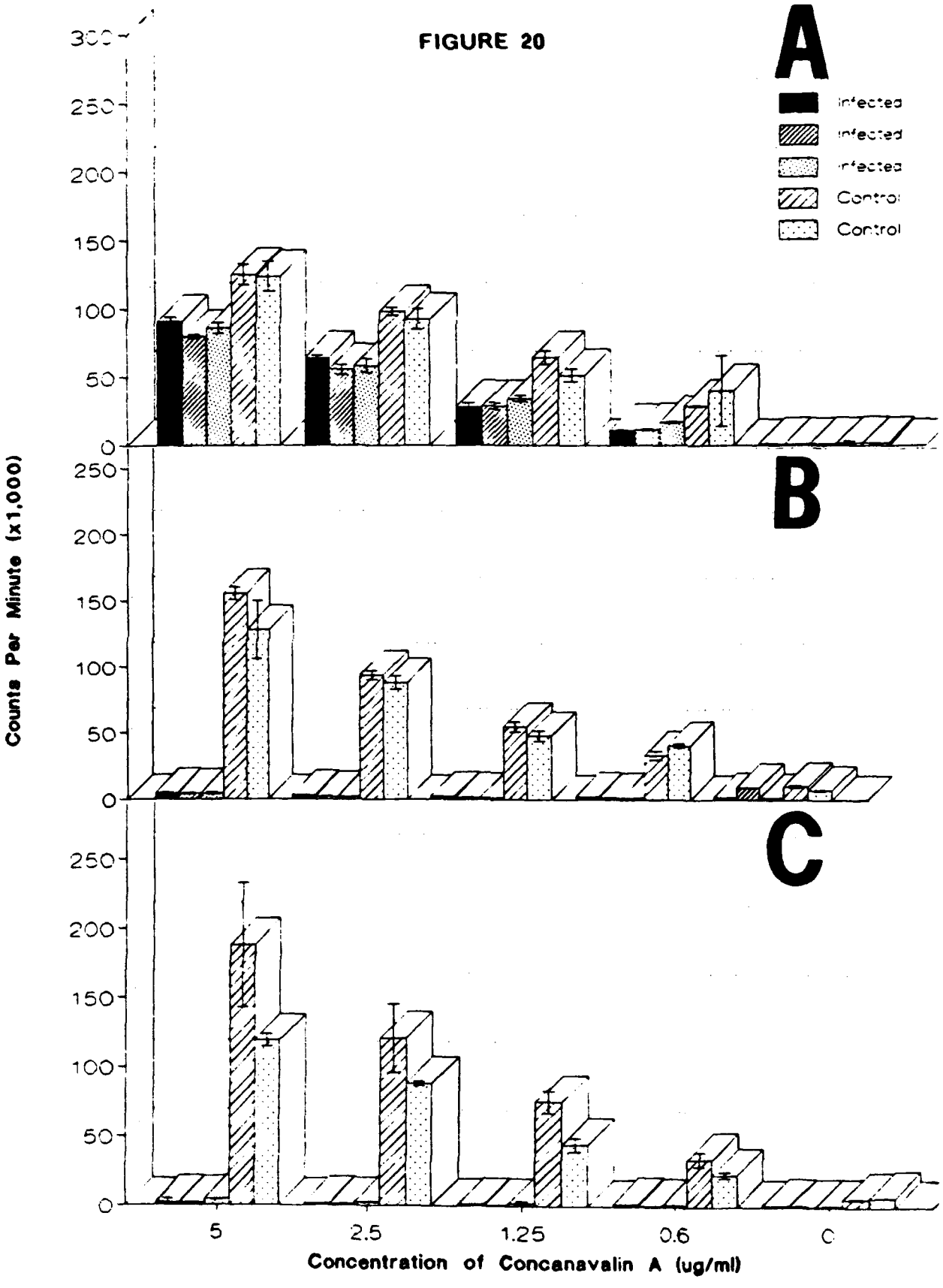
A
B
C
Infected
Infected
Infected
Control
Control
300
250
200
150
100
50
0
Counts Per Minute (x1,000)
5
2.5
1.25
0.6
0
Concentration of Concanavalin A (ug/ml)

FIGURE 21

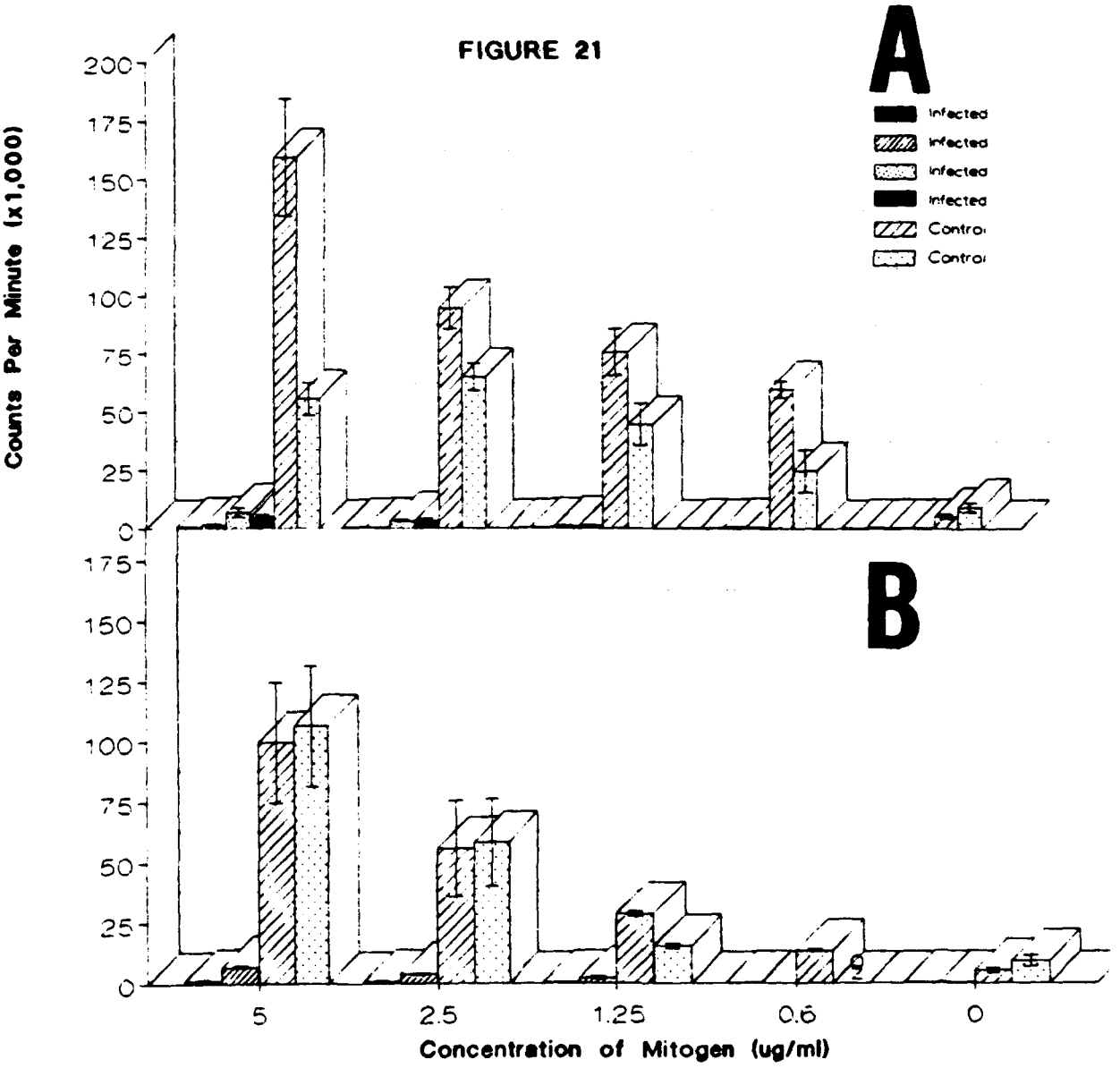
A
B
Infected
Infected
Infected
Infected
Control
Control
Counts Per Minute (x1,000)
200
175
150
125
100
75
50
25
0
5
2.5
1.25
0.6
0
Concentration of Mitogen (ug/ml)

FIGURE 22

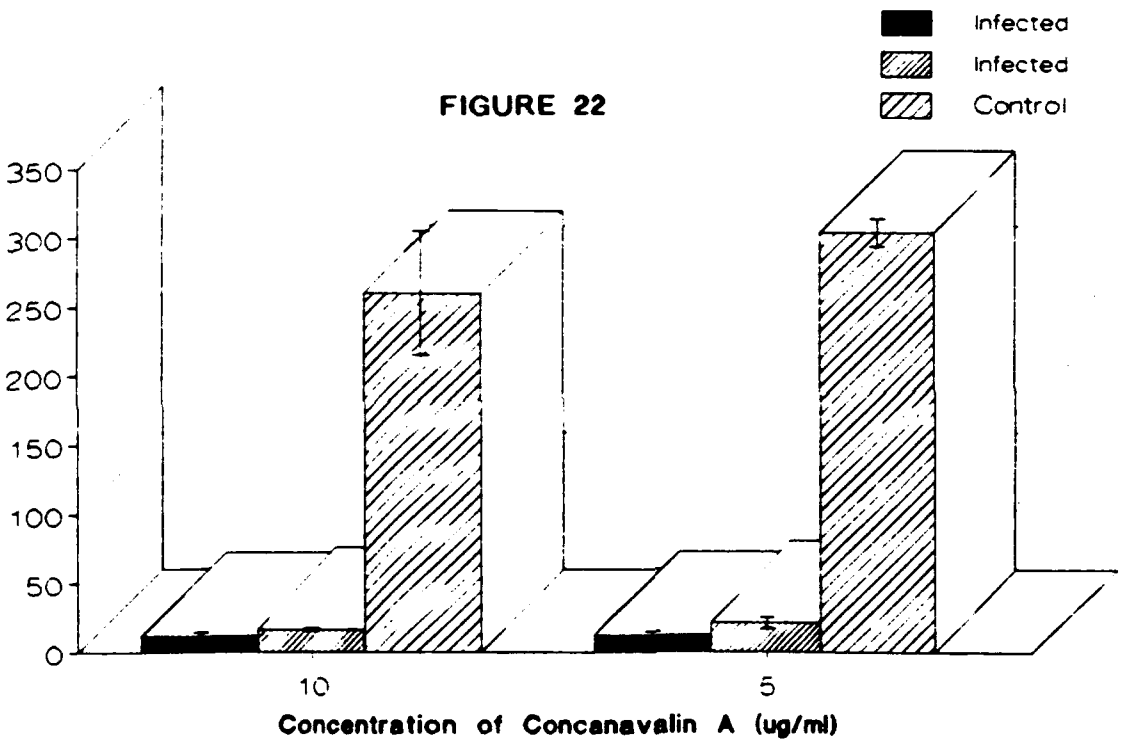
Infected
Infected
Control
350
300
250
200
150
100
50
0
10
5
Concentration of Concanavalin A (ug/ml)

FIGURE 23

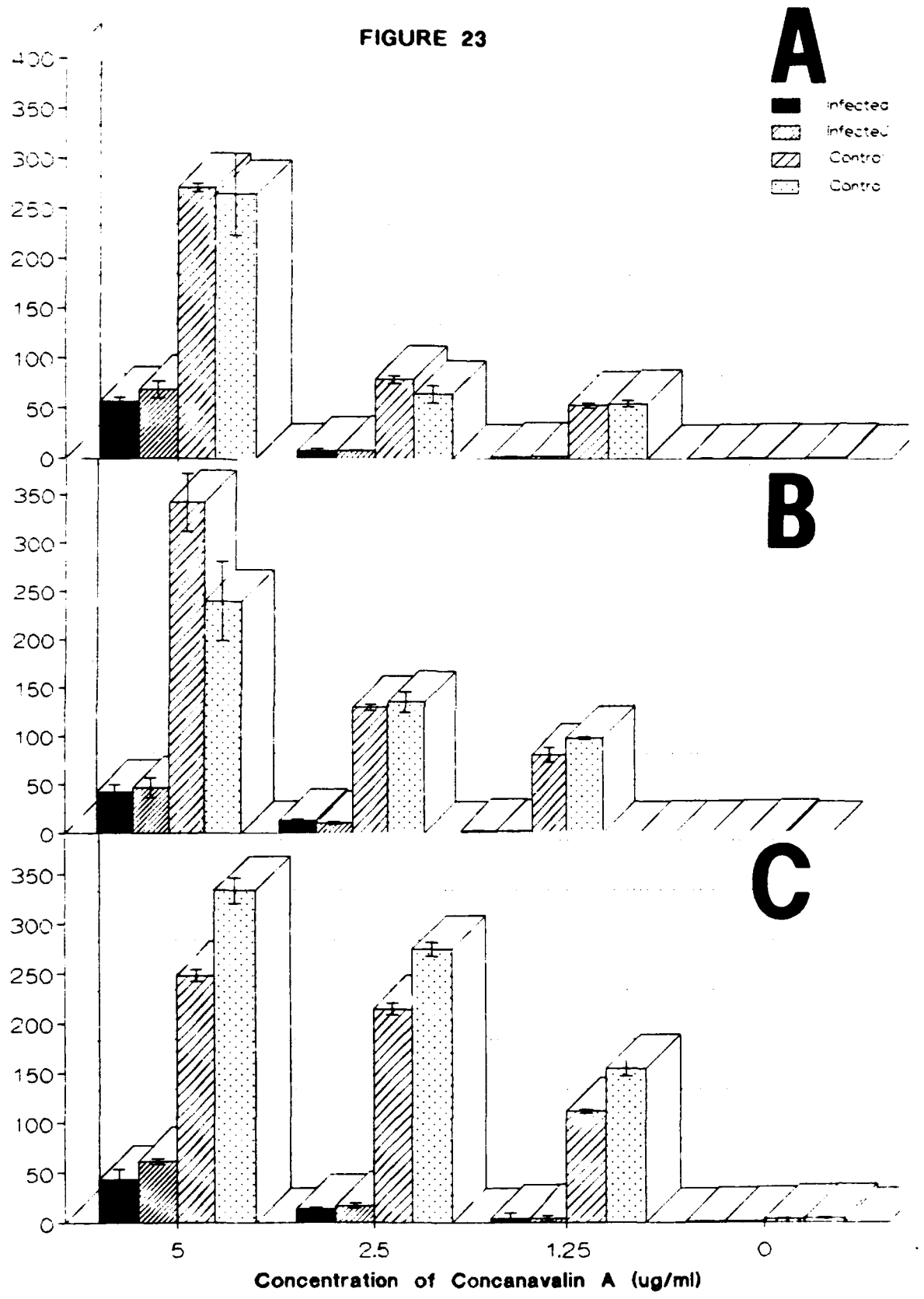
A
B
C
Infected
Infected
Control
Control
400
350
300
250
200
150
100
50
0
5
2.5
1.25
0
Concentration of Concanavalin A (ug/ml)

TABLE 1 adPIC INFECTION IN OUTBRED GUINEA PIGS

GP#	outcome	day of death	lesions	log spleen viral titer (PFU/G)	bact. culture
1	20% wt. loss	9	n.d.	8.079	neg. (lung, spleen)
2	29% wt loss, paralysis	12	Lymph node necrosis, pneumonitis, ?myelitis	8.114	neg (spleen)
3	27% wt loss, paralysis	13	enteritis, adrenal microabscesses	7.255	pos.*♦■ (spleen)
4	26% wt loss	12	pneumonitis, enteritis	8.114	n.d.
5	25% wt loss	11	renal tubular casts	7.653	n.d.
6	27% wt loss, paralysis	13	pulm. edema, endomyocarditis	7.279	pos.*♦ (spleen)
7	22% wt loss, paralysis	12	pneumonitis	8.322	neg. (spleen)
8	26% wt loss	12	n.d.	n.d.	n.d.
9	n.d.	16	n.d.	n.d.	n.d.
10	19% wt loss	12	n.d.	n.d.	n.d.
11	27% wt loss	11	n.d.	8.519	n.d.
12	28% wt loss, paralysis	13	pneumonitis, renal casts, spleen necrosis, myocarditis	8.380	pos.■ (spleen)

TABLE 2

Dose response of strain 13 guinea pigs to PIC 3739

viral inoculum dose (pfu)	# of animals in group	mean day of onset of fever	mean duration of fever (days)
$5x10^4$	4	6.5 ± 1.0	3.5 ± 1.0
$5x10^3$	4	7.0 ± 0	3.8 ± 0.9
$5x10^2$	4	7.5 ± 1.0	4.5 ± 1.0
$5x10^1$	4	7.5 ± 1.0	6.0 ± 0

TABLE 3

SERUM TNF IN GUINEA PIGS INFECTED WITH PIC STRAINS

	adPIC		PIC3739	
p.i. day	TNF pg/ml	#pos/n	TNF pg/ml	#pos/n
3	2.6 ± 8.9	1/12	0 ± 0	0/3
6	5.1 ± 17.3	2/12	0 ± 0	0/4
7	21.3 ± 6.7	2/2	n.d.	0/0
11	19.2 ± 51.4	§4/14	0 ± 0	0/9
12	0.3 ± 0.7	†2/7	n.d.	0/0
*15	73.5 ± 35.7	⧫8/8	0 ± 0	0/6
18	0 ± 0	‡0/1	n.d.	0/0

TABLE 4

RELATIVE AMOUNTS OF TNF mRNA IN GP ORGANS DURING PIC INFECTION

Organ or cells	Fold increase in TNF mRNA after stimulation with:	
	LPS	adPIC
Liver	2.1	1.3
Spleen	1.0	2.7
Heart	1.4	0.8
Macrophages	29	n.d.

Table 5

Partial Purification of Guinea Pig TNF

purification step	total protein (mg)	total activity (U)	% recovered activity	specific activity (U/mg)	purification factor
1-PEC spnt	3250	7.9×10^6	-------	2.4×10^3	------
2-amicon	370$^{\odot}$	7.8×10^7	980	2.1×10^5	87.5
3-pool*	1368	5.0×10^7	64	3.6×10^5	150
4-centricon	103.6$^{\odot}$	9×10^7	180	8×10^5	333
5-millipore	7.5	1.5×10^5	0.17$^{\ominus}$	2×10^4	8.3
6-HPLC*$^{\circledcirc}$	.034	$7.8 \times 10^{4\circledcirc}$	370	2.2×10^6	917

Concentration and purification of supernatants from LPS-stimulated guinea pig peritoneal macrophges (PEC) were performed as described in the text. Purification steps have been abbreviated as follows:

1) starting PEC supernatants
2) material concentrated by ultrafiltration through a YM-10 membrane
3) material from step #2 pooled with a small amount of residual unconcentrated supernatant
4) material from step #3 further concentrated by ultrafiltration in a centricon-10 microconcentrator (Amicon)
5) concentrated material passed through a 0.22 micron, low protein-binding Durapore filter to remove particulate matter
6) pooled HPLC fractions containing bioactive TNF

$^{\circledcirc}$ 1 mg protein from step #5 loaded on HPLC column
$^{\ominus}$ large decline in activity reflects removal of cell membrane material which probably was rich in TNF
$^{\odot}$ decrease in total protein reflects ultrafiltration of low molecular weight amino acids which are detected in the BCA assay
* discontinuities in purification scheme, i.e. total TNF of this step cannot be compared directly to step above on the chart

TABLE 6.

HISTOLOGIC FEATURES ASSOCIATED WITH LETHALITY IN PIC INFECTION

organ	lesion	group 1 (moribund)	group 2 (recovering)	control
spleen	MZN	7/7 (100%)	5/11 (45.4%)	0/1
	RPN>2+	6/7 (85.7%)	1/11 (9.1%)	0/1
LN	H	3/3 (100%)	6/6 (100%)	---
	N	1/3 (33.3%)	0/6 (0%)	---
Liver	F>2+	6/7 (85.7%)	5/8 (62.5%)	0/1
	PI>2+	1/7 (14.3%)	4/8 (50%)	0/1
	LI/HN 3+	3/7 (42.9%)	4/8 (50%)	0/1
Lung	IP>1+	1/6 (16.7%)	2/8 (25%)	0/1
Gut	FM>1+	7/7 (100%)	2/8 (25%)	0/1
	CY>2+	4/7 (57.1%)	0/8 (0%)	0/1
	VF>2+	6/7 (85.7%)	0/8 (0%)	0/1
Spleen	weight	0.66 ± 0.11g	1.17 ± 0.06g	0.60 ± 0.02g

TABLE 7
PROTECTION OF GUINEA PIGS AGAINST LETHAL ADPIC INFECTION BY IMMUNIZATION WITH PIC3739

Immunizing dose (PV3739) (pfu)	# survived/# challenged
$5x10^4$	2/2
$5x10^3$	2/2
$5x10^2$	2/2
$5x10^1$	2/2

500 gram male strain 13 guinea pigs were inoculated subcutaneously with the indicated dose of PIC3739, and challenged intraperitoneally 30 days later with 10^4 pfu of adPIC.

TABLE 8
PIC 3739 immunization
IFA serology

GP#	immunizing dosePIC3739 (pfu)	challenged (10,000 pfu adPV)	anti-adPIC titer	anti-3739 titer
1	50,000	+	400	>3200
2	50,000	+	400	>3200
3	50,000	-	400	3200
4	50,000	-	100	1600
5	5,000	+	200	1600
6	5,000	+	100	800
7	5,000	-	800	1600
8	5,000	-	200	800
9	500	+	800	>3200
10	500	+	400	800
11	500	-	400	800
12	500	-	400	1600
13	50	+	800	>3200
14	50	+	800	>3200
15	50	-	>3200	>3200
16	50	-	800	1600
normal GP	0	-	<100	<100

Indirect immunofluorescence assays were performed using acetone-fixed adapted PV (adPV)-infected or strain 3739-infected Vero cell antigen dots and FITC goat anti-guinea pig IgG (H+L) (Kierkegaard-Perry). Test sera were applied in serial 2-fold dilutions. Titers are reported as the reciprocal of the highest dilution showing specific fluorescence.

TABLE 9

Effect of Adherent Cells from Control and Infected Guinea Pigs on Lymphocyte Responses to Concanavalin A[a]

			Source of Lymphocytes		
		Con A[b]	None	Infected	Control
urce of acro-ages	None	+	ND	5,300[c]	962
	None	-	ND	ND	604
	Infected	+	88	2,692	540,643
	Infected	-	117	404	606
	Control	+	178	3,704	442,996
	Control	-	70	372	841

[a] Lymphocytes and macrophages were isolated as described in materials and methods, from infected and control animals, 11 days after inoculation with either 2 x 10^4 PFU of adPIC virus or RPMI 1640. Different combinations of each cell type were incubated in the presence or absence of 10 μg/ml concanavalin A for 72 hours.

[b] Designates presence or absence of 10 μg/ml concanavalin A in the culture.

[c] Proliferation was determined by ^{3}H-labeled thymidine incorporation. Values are represented as means of the counts per minute determined for quadruplicate cultures. All standard deviations were $<10\%$ of the mean, or <200 counts per minute.

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MACROPHAGES AND MONOKINES IN GUINEA PIG ARENAVIRUS DISEASE J. Aronson, N. Herzog, and T. Jerrells, Univ. of Texas Medical Branch, Galveston, TX, USA and Louisiana State Univ. Med. Center, Shreveport, LA, USA.

Pichinde virus (PIC) is a reticuloendothelial tropic New World arenavirus. A guinea pig (GP) passage-adapted strain (adPIC) is highly virulent for inbred GPs, and forms the basis for a model of human Lassa Fever. Guinea pig adPIC infection is associated with marked weight loss, fever, and death by about day 12 post-inoculation. Tumor necrosis factor (TNF) bioactivity is found in serum in 30-100% of animals late in the course. TNF activity is also present in spleen homogenates, peaking at day 6 and declining by day 11. There is a parallel 2-3 fold increase in TNFα mRNA in spleen tissue at day 6. A related PIC strain, CoAN 3739 (PIC3739) (non-passage adapted) has reduced virulence for GPs, and does not elicit TNF levels in serum. Studies on explanted macrophage populations from infected GPs indicate that peritoneal macrophages (PM) from adPIC infected animals overproduce TNF to LPS stimulus compared to macrophages from PIC3739 infected GPs or uninfected control macrophages. Splenic macrophages (SM) in adPIC infection appear suppressed in terms of TNF production after LPS stimulation. adPIC replicates faster and to higher titers than PIC3739 both in vivo (based on organ viral titers), and after in vitro infection of PM. A marked decrease in the number of "resident" PM is seen late in the course of adPIC infection, but not in PIC3739 infection. Whereas scattered hepatic inflammatory lesions and steatosis are seen in animals infected with either virus strain, fibrinoid necrosis of macrophage-rich regions of the spleen and pronounced macrophage infiltration of the lamina propria of the small bowel are seen only in lethal infections with adPIC. These data suggest that features inherent in the macrophage-PIC interaction, including induction of monokines and frank viral cytopathic effect on target macrophages, contribute to pathogenesis of arenavirus disease in guinea pigs. (Supported by James W. McLaughlin Fellowship Fund and U.S. Army Contract DAMD17-

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THE ROLE OF TNF IN PATHOGENESIS OF ARENAVIRUS DISEASE IN GUINEA PIGS: COMPARISON OF TWO PICHINDE VIRUS STRAINS. J. Aronson, N. Herzog, & T. Jerrells. (SPON: D.H. Walker). Univ. of Texas Medical Branch, Galveston, TX 77550 and Louisiana State Univ. Medical Center, Shreveport, LA 71130.

Pichinde virus (PIC) is a reticuloendothelial-tropic New World arenavirus. A passage adapted PIC strain (adPIC) is highly virulent for guinea pigs (GP). The related strain CoAn-3739 (PIC3739) has greatly reduced virulence in strain 13 guinea pigs. Infection with adPIC, but not PIC3739, was associated with detectable serum tumor necrosis factor (TNF). Peritoneal (PEC) or splenic macrophages (SM) explanted from adPIC infected, but not PIC3739 infected, GP spontaneously secreted low levels of TNF. PEC from adPIC infected animals showed a marked augmentation of LPS-inducible TNF production compared to uninfected controls. Increase in LPS-inducible TNF production was also seen in PEC infected *in vitro* with PIC. In focus forming assays, the two virus strains had similar rates of initiation of macrophage infection *in vitro*. Subsequently adPIC produced higher levels of extracellular virus than did PIC3739. These data suggest that the virulence of adPIC is based on replication advantage in the chief target cell. Priming of macrophages for TNF production parallels viral burden. This sensitization occurs preferentially in macrophages infected with the faster replicating adPIC strain. The resulting overproduction of TNF may contribute to pathophysiologic sequelae.

(Supported by James W. McLaughlin Fellowship Fund and Army Contract DAMD17-90-Z-0011).

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MONOKINE PRODUCTION AND PATHOGENESIS OF ARENVIRUS INFECTION IN GUINEA PIGS. J. Aronson, V. Brown, T. Jerrells, Dept. of Pathology, UTMB, Galveston, TX 77550

Pichinde Virus (PV) is a New World arenavirus that comprises a rodent model for human Lassa fever. Based on data showing that macrophages are targets for PV infection, we are examining the roles of monokines in pathogenesis and immunity. Strain 13 guinea pigs develop fever, profound cachexia (20% loss of body weight), and death in response to intraperitoneal inoculation with PV. Strain 2 guinea pigs given the same dose develop an earlier and more dramatic febrile response, but do not show the same degree of cachexia and go on to recover. Strain 13 guinea pigs have 10 fold higher viral titers in blood and spleen than their strain 2 counterparts. Cultured peritoneal and splenic macrophages were examined by immunofluorescence for the presence of viral antigen. Approximately 15-25% of peritoneal macrophages from both strains were positive for viral antigen. Macrophages from infected guinea pigs did not constitutively produce tumor necrosis factor (TNF), but were able to express TNF after in vitro stimulation with LPS. Moribund animals had detectable levels of TNF in their serum. These data support the hypothesis that TNF, perhaps in combination with other cytokines, plays a role in the pathogenesis of PV infection. (Supported by the James W. McLaughlin Fellowship Fund.)

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DIMINISHED LYMPHOCYTE PROLIFERATION AND IL-2 PRODUCTION BY LYMPHOCYTES FROM GUINEA PIGS INFECTED WITH PICHINDE VIRUS

V.K. Brown, J.F. Aronson, T.R. Jerrells, Dept. of Pathology, University of Texas Medical Branch, Galveston, TX 77550

Pichinde virus infection of strain 13 guinea pigs is characterized by cachexia (>20% weight loss), fever and death. Virus-induced immunosuppression may contribute to the severity of the infection. The present experiments were designed to test the overall immune status of infected animals. Analysis of mitogen-induced IL-2 production and lymphocyte proliferation (LP) of splenic, lymph node and peripheral blood lymphocytes isolated from infected animals was performed. Strain 13 guinea pigs were infected with 5 x 10^4 plaque forming units of Pichinde virus given intraperitoneally. At various times after infection, mitogen-induced LP and IL-2 production were evaluated. LP was measured after 72 hours in culture by tritiated thymidine uptake, and levels of IL-2 in 18 to 20 hour-supernatants were determined using the IL-2-dependent cell line, CTLL. It was found that LP to T-cell mitogens, concanavalin A (Con A) or phytohemagglutinin (PHA), was progressively diminished as the infection advanced, with maximal immunosuppression present on day 13 post infection. Similar decreases in IL-2 production were noted. These results indicate that Pichinde virus infection is associated with an immunosuppression in susceptible animals. (This work supported in part by the Zelda Zinn Casper Endowed Scholars Program)

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